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(54) Title: ENVIRONMENTALLY REGULATED GENES OF *STREPTOCOCCUS SUIS*

(57) Abstract: The invention relates to the field of diagnosis of and vaccination against Streptococcal infections and to the detection of virulence markers of Streptococci. The invention provides a method for modulating virulence of a *Streptococcus* comprising modifying a genomic fragment of said *Streptococcus* wherein said genomic fragment comprises at least a functional part of a fragment identifiable by hybridisation in *Streptococcus suis* to a nucleic acid or fragment thereof.

Tittle: Environmentally regulated genes of *Streptococcus suis*.

The invention relates to the field of diagnosis of and vaccination against Streptococcal infections and to the detection of virulence markers of Streptococci.

Streptococcus species, of which there are a large variety causing infections in domestic animals and man, are often grouped according to Lancefield's groups. Typing according to Lancefield occurs on the basis of serological determinants or antigens that are among others present in the capsule of the bacterium and allows for only an approximate determination, often bacteria from a different group show cross-reactivity with each other, while other Streptococci can not be assigned a group-determinant at all. Within groups, further differentiation is often possible on the basis of serotyping; these serotypes further contribute to the large antigenic variability of Streptococci, a fact that creates an array of difficulties within diagnosis of and vaccination against Streptococcal infections.

Lancefield group A *Streptococcus* (GAS, *Streptococcus pyogenes*), are common with children, causing nasopharyngeal infections and complications thereof. Among animals, especially cattle are susceptible to GAS, whereby often mastitis is found.

Lancefield group B *Streptococcus* (GBS) are most often seen with cattle, causing mastitis, however, human infants are susceptible as well, often with fatal consequences. Group B streptococci (GBS) constitute a major cause of bacterial sepsis and meningitis among human neonates born in the United States and Western Europe and are emerging as significant neonatal pathogens in developing countries as well.

Lancefield group C infections, such as those with *S. equi, S. zooepidemicus, S. dysgalactiae*, and others are mainly seen with horse, cattle and pigs, but can also cross the species barrier to humans.

Lancefield group D (*S. bovis*) infections are found with all mammals and some birds, sometimes resulting in endocarditis or septicaemia.

Lancefield groups E, G, L, P, U and V (*S. porcinus, S, canis, S. dysgalactiae*) are found with various hosts, causing neonatal infections, nasopharyngeal infections or mastitis.

Within Lancefield groups R, S, and T, (and with ungrouped types) *S. suis* is found, an important cause of meningitis, septicemia, arthritis and sudden death in young pigs. Incidentally, it can also cause meningitis in man.

Ungrouped *Streptoccus species*, such as *S. mutans*, causing carries with humans, *S, uberis*, causing mastitis with cattle, and *S. pneumonia*, causing major infections in humans, and *Enterococcus faecilalis* and *E. faecium*, further contributed to the large group of Streptococci. *Streptococcus pneumoniae* (the pneumococcus) is a human pathogen causing invasive diseases, such as pneumonia, bacteraemia, and meningitis.

Little is known about the pathogenesis of the disease caused by Streptococci. Various cellular components, such as muramidase-released protein (MRP) extracellular factor (EF) and cell-membrane associated proteins, fimbriae, haemagglutinins, and haemolysin have been suggested as virulence factors. However, the precise role of these protein components in the pathogenesis of the disease remains unclear. It is however, well known and generally accepted that the polysaccharidic capsule of various Streptococci and other gram-positive bacteria plays an important role in pathogenesis. The capsule enables these micro-organisms to resist phagocytosis and is therefore regarded as an important virulence factor or marker.

In particular, *Streptococcus suis* is an important cause of meningitis, septicemia, arthritis and sudden death in young pigs. It can also cause meningitis in man. Attempts to control the disease are still hampered by the lack of sufficient knowledge about the pathogenesis of the disease and the lack of effective vaccines and sensitive diagnostic methods.

So far, 35 serotypes of S. *suis* are described. Virulence of *S. suis* can differ within and among serotypes. Worldwide *S. suis* serotype 2 is the most frequently isolated serotype. Within *S. suis* serotype 2, pathogenic, weak-pathogenic and non-pathogenic strains can be found. The pathogenic strains cause severe clinical signs of disease in pigs and large numbers of bacteria can be reisolated from the central nervous system (CNS) and the joints after experimental infection. The weak-pathogenic strains cause only mild clinical signs of disease and only infrequently bacteria can be reisolated from the CNS and the joints after experimental infection. The non-pathogenic strains are completely avirulent in young pigs after experimental infection.

The 136-kDa muramidase-related protein (MRP) and the 110-kDa extracellular factor (EF) are generally considered as important virulence markers for *S. suis* serotype 2 strains isolated in Europe and the United States. However, differences in virulence between pathogenic, weak-pathogenic and non-pathogenic strains cannot exclusively be explained by differences in their MRP and EF expression patterns. In addition, it is known that the capsule of *Streptococcus suis* serotype 2 is an important virulence factor. However, since both pathogenic, weak-pathogenic and non-pathogenic strains seemed to be fully encapsulated after growth *in vitro* and *in vivo*, it is not likely that the level of encapsulation of these fully encapsulated strains is associated with their difference in virulence.

The invention provides a method for modulating virulence of a *Streptococcus* comprising modifying a genomic fragment of said *Streptococcus* wherein said genomic fragment comprises at least a functional part of a fragment identifiable by hybridisation in *Streptococcus suis* to a nucleic acid or fragment thereof as shown in Fig 6 and obtaining a clone wherein said genomic fragment has been modified. In one preferred embodiment, said genomic fragment comprises at least a functional part of a gene expression of which in *S. suis* can be environmentally regulated by iron-restricted conditions. In another preferred embodiment, said genomic fragment comprises at least a functional part of a gene which is expressed in a with a wild-type *S. suis* infected pig (*in vivo*). In a most preferred embodiment, selection under iron-restricted conditions is combined with selection *in vivo*. It is especially preferred that that said gene encodes a fibronectin/fibrinogen binding protein. The method as provided herein is especially useful for modulating virulence of a *Streptococcus suis*, and preferably comprises functionally deleting the expression of at least said functional part of said gene by said *Streptococcus*. By functionally deleting is meant any technique known in the art (such as allowing for a deletion, insertion, mutation or the occurrence of a frame-shift in the open-reading frame of the respective gene), that is instrumental in hampering or inhibiting the expression of a gene-product (be it mRNA and/or protein) of said gene. The invention thus provides a clone of a Streptococcus obtained or obtainable by a method according to the invention.

To get insight in the differences between pathogenic, weak-pathogenic and non-pathogenic strains or clones, that determined their difference in virulence, the

invention provides the identification of environmentally regulated genes of *Streptococcus suis* by iron-restricted conditions and by experimental infection of piglets. Eighteen unique iron-restricted induced (*iri*) genes and 22 unique *in vivo* selected (*ivs*) genes of *S. suis* were found. None of the *ivs* genes was exclusively expressed *in vivo*. Four *iri* genes were identical to four *ivs* genes selected in piglets. Two *ivs* genes were similar to genes for putative virulence factors. One of these *ivs* genes was identical to the *epf* gene of virulent *S. suis* serotype 2 strains and the other showed homology to a gene encoding a fibronectin-binding protein of *Streptococcus gordonii*. As a further studied example, the invention provides a study of the chracteristics of fibronectin- and fibrinogen binding protein of Streptococcus suis (FBPS) and its gene as identified herein. The ability of bind fibronectin, either in fluid phase or immobilized onto a surface is thus a property of S. suis, and is one of the mechanisms S. suis uses for attachment to and invasion of host cells. Therefore FBPS is an important virulence factor. The gene encoding this protein was identified using an in vivo selection system in pigs as provided herein, again, showing an important role of the protein in vivo. This finding was supported by the observation that isogenic FBPS mutants, herein also provided, of S. suis are attenuated in pigs. Surprisingly, FBPS bound to fibronectin as well as to fibrinogen but did not show the structural characteristics of the fibronectin binding proteins most commonly described, explaining why it has not been found earlier. Most fibronectin-binding proteins described to date are large cell surface proteins with predicted sizes of 60-100 kDa with very similar structural organisations. The proteins contain a N-terminal signal sequence as well as the cell wall signalling sequence (LPXTGE). The Fn-binding sites consists of 30-42 amino acid long motifs, repeated 3-4 times. In particular, we provide herein the first fibronectin- and fibrinogen binding protein of *S. suis*.

The gene encoding FBPS was cloned and sequenced and FBPS was purified. Binding of FBPS to human fn and fgn was shown. FBPS was shown to be involved in the colonization of the organs specific for an *S. suis* infection in piglets, but not in the colonization of *S. suis* on the tonsils of piglets.

Many streptococci and staphylococci have several different fibronectin- and/or fibrinogen-binding proteins, most of which are very large, about 130 kDa. Until now, *S. pyogenes* is the only organism having a large as well as a smaller (54 kDa) FnBP. The existence of more than one FnBP explains why in some organisms isogenic mutants defective in only one of the FnBPs can still bind to fn and/or fgn can be further attenuated *in vivo* in relation to fibronectin binding.

The role of FBPS in the pathogenesis of *S. suis* was studied in an experimental infection model in piglets. Since we were unable to determine a LD_{50} values for the mutant clones, it being found that no lethal dose could be established using normally used numbers of bacteria, it was decided to compare the virulence of the isogenic FBPS clone to the wild-type *S. suis* in a competitive infection assay in piglets. This kind of co-colonization experiments have been successfully applied to determine the virulence of mutants of *Actinobacillus pleuropneumoniae* in piglets. The data clearly showed that the mutant clone was capable of colonizing the tonsil as efficiently as the wild-type. This strongly indicates that FBPS is not involved in the colonization of the tonsil. The data also indicated that FBPS does play a role in the colonization of specific organs, since in the competition assay joints and the CNS were more efficiently colonized by wild-type than by mutant bacteria. In addition, higher numbers of wild-type bacteria were re-isolated from the specific organs compared to the numbers of mutant bacteria, indicating that the mutant clone is attenuated *in vivo*. Although the number of pigs used for this experiment was low, these data

indicate that the FBPS mutant is less virulent than the wild-type strain. We were able to demonstrate that FBPS reacted with a convalescent serum of a pig that survived an *S. suis* infection. Therefore FBPS is immunogenic in pigs, and this finding clearly demonstrates that FBPS of *S. suis* is expressed under *in vivo* conditions. We showed that the *fbps* gene was present in all known serotypes of *S. suis* (except for two), as well as in all three phenotypes of serotype 2. This suggests that the *fbps* gene is present among most serotypes. However, the expression of FBPS in all serotypes and phenotypes was not studied. Therefore it is possible, that although all strains, except for serotypes 32 and 34, possess the *fbps* gene, not all strains express FBPS. Based on the facts that FBPS is immunogenic in pigs, and that the *fbps* gene is present in all prevailing *S. suis* serotypes except for 2, FBPS is a very attractive candidate for a cross-protective vaccine against essentially all serotypes. An attractive option is to use the mutant strain 10)FBPS in said vaccine, which mutant is possible further attenuated by deleting one or more virulence factors as identified herein, in another embodiment this vaccine is based on purified FBPS protein or an antigenic part thereof with a suitable adjuvant. In short, to further validate a method for identifying a virulence factor as identified herein we further investigated the role of the fibronectin-/fibrinogen-binding protein (FBPS) in the pathogenesis of *S. suis* serotype 2 in piglets. The complete gene encoding FBPS from *S. suis* serotype 2 was cloned in *E. coli* and sequenced. The occurrence of the gene in various serotypes was analyzed by hybridization studies. The FBPS protein was expressed in *E. coli*, purified and binding to human fibronectin and fibrinogen was demonstrated. The induction of antibodies in piglets was studied upon infection. An isogenic mutant unable to produce FBPS was constructed and the virulence of the wild-type and mutant strains was compared in a competitive

infection model in young piglets. Organ cultures showed that FBPS was not required for colonization of the tonsils but that FBPS played a role in the colonization of the specific organs involved in an *S. suis* infection. Therefore, the FBPS mutant was considered as an attenuated mutant which is very useful in a vaccine. Alternatively, a vaccine is used that mainly consists of the FBPS protein or at least of an antigenic part thereof, such that an FBPS-specific antibody or T-cell response in pigs is developed after vaccination with the FBPS or antigenic part thereof.

Two additional *ivs* genes showed homology to environmentally regulated genes previously identified by using an *in vivo* expression technology (IVET) selection in other bacterial species. One of these showed similarity to the *agrA* gene of *Staphylococcus aureus*, a key locus involved in the regulation of numerous virulence proteins.

Thereby, the invention also provides a method for assaying virulence of a *Streptococcus* comprising assaying a genomic fragment of said *Streptococcus* wherein said genomic fragment comprises at least a functional part of a fragment identifiable by hybridisation in *Streptococcus suis* to a nucleic acid or fragment thereof as provided herewith.

The invention also provides a vector comprising a nucleic acid according to the invention, and a host cell comprising a nucleic acid or a vector according to the invention. Such a host cell preferably comprises an easily modifiable organism such as E. coli, however, other host cells, such as recombinant *Streptococcus* comprising a vector or nucleic acid according to the invention are herein also provided.

Furthermore, the invention provides a vaccine comprising a nucleic acid or a vector or a host cell according to the invention, and use of such a vaccine in the prevention and/or treatment of Streptococcal infections.

Also provided is a protein or fragment thereof encoded by a nucleic acid according to the invention, such as a protein encoded by a nucleic acid or fragment thereof disclosed herein or functional, i.e. antigenic fragment thereof. The invention also provides an antibody directed against a protein or fragment thereof according to the invention and an antigen reactive with such an antibody,

for example comprising a protein or fragment. Such a protein or fragment thereof need not be obtained by recombinant means only, synthesis of peptides, according to their amino acid sequence, is equally well possible. Such antigens and antibodies as provided herein can be used in a diagnostic test comprising an antibody according to the invention, or within a vaccine or diagnostic test comprising an antigen according to the invention. Such vaccines and diagnostic tests can be used in the field of the field of diagnosis of and vaccination against Streptococcal infections and for the detection of virulence markers of Streptococci.

LEGENDS

FIG. 1.

Schematic presentation of the procedure used to clone the *fbps* gene of *S. suis* serotype 2 and the construction of an insertional knock-out mutant in *S. suis* serotype 2. A 5 kb *Eco*RI fragment was cloned in pGEM7Zf(+), yielding pFBPS7-46. In pFBPS7-47, the 382 bp *Sal*I-*Sal*I fragment of pFBPS7-46 was replaced by 1.2 kb spectinomycin resistance gene, after the vector was made blunt, to obtain an insertional knock-out of *fbps*. *ivs-31*: *in vivo* selected gene 31.

FIG 2.

Purity and immunogenicity of FBPS purified under native conditions. SDS-PAGE analysis with SYPRO orange, a non-specific protein-staining (panel A) and Western blot analysis with a monoclonal antibody against the 6 x HIS tag (panel B) of 4 :l of *E. coli* M15 [pQE-30-pREP4-FBPS] lysate (lanes 1) and 165 ng of purified FBPS (lanes 2). Convalescent serum raised against *S. suis* strain 10 was used to test immunogenicity of FPBS present in 4 :l of *E. coli* M15 [pQE-30-pREP4-FBPS] lysate and 0.5 :g of purified FBPS (Panel C, lanes 1 and 2). Arrowhead, 64 kDa FPBS; Mw, molecular weight marker.

FIG 3 .

Binding studies with purified FBPS. Panels A and B were probed with 5 :g/ml of fn (A) or fgn (B). Lanes 1 contain 500 ng of purified FBPS, lanes 2 contain 500 ng of BSA. Panels C and D, lanes 3 and 4 contain 500 ng of purified FBPS. Lanes 3 were probed with 20 :g/ml of fn (C) or fgn (D), lanes 4 were only incubated with conjugate without fn or fgn. Panels E and F were probed with 20 :g/ml of fn (E) or fgn (F). Lanes 5 contain 1.8 :g of purified FBPS digested with enterokinase,

lanes 6 contain 500 ng of purified FBPS. The closed arrowhead indicates 64 kDa FBPS; the open arrowhead indicates approximately 55 kDa FBPS without 6 x HIS.

FIG. 4

Distribution of *fbps* among various *S. suis* serotypes. 1 :g of chromosomal DNA was spotted onto nitrocellulose membrane and hybridized with a ^{32}P-labelled *fbps* probe. Serotypes were spotted as indicated. S10: *S. suis* serotype 2, MRP$^+$EF$^+$; T15: *S. suis* serotype 2 MRP$^-$EF$^-$; S17: *S. suis* serotype 2 MRP$^+$EF*.

FIG. 5

Efficiency of colonization of wild-type and mutant bacteria on various organs of infected pigs. Panel A depicts colonization of the wild-type strain 10 and the mutant strain 10)FBPS of the tonsils. υ tonsil pig no. 4664; ν tonsil pig no. 4665; σ tonsil pig no 4666; λ tonsil pig no. 4668. Panel B depicts colonization of the specific organs. open and closed υ pus from joints pig no. 4664; σ pus from joint pig no. 4666; λ CNS pig no. 4668. Each dot represents the numbers of wild-type or mutant bacteria isolated from one particular organ, from one piglet.

Fig. 6 A

Nucleotide sequence of iri 1, 6, 22 as identified herein.

Fig. 6 B

Nucleotide sequence of iri 10, 20 as identified herein.

Fig. 6 C

Nucleotide sequence of iri 11 as identified herein.

Fig. 6 D

Nucleotide sequence of iri 13, 15, 27 as identified herein.

Fig. 6 E

Nucleotide sequence of iri 14 as identified herein.

Fig. 6 F

Nucleotide sequence of iri 16 as identified herein.

Fig. 6 G

Nucleotide sequence of iri 18 as identified herein.

Fig. 6 H

Nucleotide sequence of iri 2 as identified herein.

Fig. 6 I

Nucleotide sequence of iri 23 as identified herein.

Fig. 6 J

Nucleotide sequence of of iri 24 as identified herein.

Fig. 6 K

Nucleotide sequence of iri 29 as identified herein.

Fig. 6 L

Nucleotide sequence of iri 3 as identified herein.

Fig. 6 M

Nucleotide sequence of iri 32 as identified herein.

Fig. 6 N

Nucleotide sequence of iri 34 as identified herein.

Fig. 6 O

Nucleotide sequence of iri 4 as identified herein.

Fig. 6 P

Nucleotide sequence of iri 7 as identified herein.

Fig. 6 Q

Nucleotide sequence of iri 8, 26 as identified herein.

Fig. 6 R

Nucleotide sequence of ivs 1 as identified herein.

Fig. 6 S

Nucleotide sequence of ivs 11 as identified herein.

Fig. 6 T

Nucleotide sequence of ivs 15 as identified herein.

Fig. 6 U

Nucleotide sequence of ivs 16 as identified herein.

Fig. 6 V

Nucleotide sequence of ivs 18 as identified herein.

Fig. 6 W

Nucleotide sequence of ivs 19 as identified herein.

Fig. 6 X

Nucleotide sequence of ivs 2, 4, 28 as identified herein.

Fig. 6 Y

Nucleotide sequence of ivs 20 as identified herein.

Fig. 6 Z

Nucleotide sequence of ivs 23, 24 as identified herein.

Fig. 6 A1

Nucleotide sequence of ivs 25 as identified herein.

Fig. 6 B1

Nucleotide sequence of ivs 29 as identified herein.

Fig. 6 C1

Nucleotide sequence of ivs 3 as identified herein.

Fig. 6 D1

Nucleotide sequence of ivs 31 as identified herein.

Fig. 6 E1

Nucleotide sequence of ivs 32, 35 as identified herein.

Fig. 6 F1

Nucleotide sequence of ivs 33 as identified herein.

Fig. 6 G1

Nucleotide sequence of ivs 34 as identified herein.

Fig. 6 H1

Nucleotide sequence of ivs 36 as identified herein.

Fig. 6 I1

Nucleotide sequence of ivs 5, 10, 12, 22 as identified herein.

Fig. 6 J1

Nucleotide sequence of ivs 6, 7, 13, 14 as identified herein.

Fig. 6 K1

Nucleotide sequence of ivs 8 as identified herein.

Fig. 6 L1

Nucleotide sequence of ivs 9, 17 as identified herein.

Detailed description

Streptococcus suis is an important cause of meningitis, septicaemia, arthritis and sudden death in young pigs (Clifton-Hadley, 1983; Vecht *et al.*, 1985). It can also cause meningitis in man (Arends & Zanen, 1988). Attempts to control the disease are still hampered by the lack of sufficient knowledge about the pathogenesis of the disease and the lack of effective vaccines and sensitive diagnostic methods. To meet these shortages it is necessary to identify the genes that are involved in the pathogenic process. So far, however, only a limited number of *S. suis* genes are known (Smith *et al.*, 1992; Smith *et al.*, 1993; Serhir *et al.*, 1997; Segers *et al.*, 1998; Smith *et al.*, 1999; and accession no. AF106927, Z95920 and A57222) and of these, only a few are putatively involved in virulence (Smith *et al.*, 1992; Smith *et al.*, 1993; Jacobs *et al.*, 1994; Gottschalk *et al.*, 1995; Segers *et al.*, 1998; Smith *et al.*, 1999). Previously, putative virulence factors have been identified after growth of the bacteria in standard laboratory media. However, it is known that many important virulence factors are environmentally regulated and are induced at specific stages of the infection process (Mahan *et al.*, 1993). Recently, several approaches have been reported that allow the identification of genes that are specifically expressed in the host. Examples are signature-tagged mutagenesis (STM) and *in vivo* expression technology (IVET; Mahan *et al.*, 1993; Camilli & Mekalanos, 1995; Hensel *et al.*, 1995; Mahan *et al.*, 1995; Mei *et al.*, 1997; Young & Miller, 1997; Chiang & Mekalanos, 1998; Coulter *et al.*, 1998; Lowe *et al.*, 1998; Polissi *et al.*, 1998; Camacho *et al.*, 1999; Darwin *et al.*, 1999; Edelstein *et al.*, 1999; Fuller *et al.*, 1999; Zhao *et al.*, 1999). In addition, important virulence proteins could also be identified by the selection of genes specifically expressed under conditions mimicking *in vivo* conditions, for example by growth in iron-restricted conditions (Litwin & Calderwood, 1993; Martinez *et al.*, 1990).

The aim of the present work is to identify virulence genes of *S. suis* by the selection of environmentally regulated genes by experimental infections of piglets and by the use of iron-restricted conditions *in vitro*. For this purpose, chromosomal DNA fragments of *S. suis* were cloned in a plasmid in front of a promoterless erythromycin-resistance gene. Subsequently, the library was used for the selection of bacteria in which erythromycin-resistance was induced under

iron-restricted conditions. In addition, we selected for erythromycin-resistant bacteria after infection of piglets with the library and treatment of the piglets with erythromycin. We used pigs instead of mice for these experiments since we recently showed that virulence of *S. suis* is different in these two animal species (Vecht *et al.*, 1997). Using this approach 18 unique iron-restriction-induced (*iri*) genes as well as 22 unique *in vivo* selected (*ivs*) genes were identified, several of which are putatively involved in virulence (Smith *et al.*, 1993; Smith *et al.*, 1996).

METHODS

Bacterial strains and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *S. suis* strains were grown in Todd-Hewitt broth (Oxoid), and plated on Columbia agar (Oxoid) containing 6% (v/v) horse blood. For the selection of genes induced inron-limited conditions, *S. suis* cells were plated on agar plates containing Todd-Hewitt medium, 5% (w/v) yeast extract and 75 μM deferoxamine mesylate (Sigma). Control plates were supplemented with 38 μM $FeSO_4.7H_2O$ (Sigma). If required, antibiotics were added at the following concentrations: 100 μg spectinomycin ml^{-1} and 1 μg erythromycin ml^{-1}. *E.coli* strains were grown in Luria broth (Miller, 1972) and plated on Luria broth containing 1.5% (w/v) agar. If required, 50 μg ampicillin ml^{-1} or 50 μg spectinomycin ml^{-1} was added.

Construction of pIVS-E. The IVS selection vector used in this study comprises a spectinomycin-resistance gene and a promoterless erythromycin-resistance gene and the origin of replication of the plasmid pWVO1 (Van der Vossen *et al.*, 1987). To construct this pIVS-E, the spectinomycin-resistance gene was amplified from pKUN19-spc (Konings *et al.*, 1987; Smith *et al.*, 1995). In a PCR reaction we used the primers 5'-TGCATGCATGGATCCATCGATTTTCGTTCG-3' and 5'-CGAGCTCGGTACCTGATTACCAATTAGAAT-3', which contained *Nsi*I and *Sa* I restriction sites at their respective 5'-ends. The PCR product obtained was digested with *Nsi*I and *Sa* I and ligated into pGKV210 (Van der Vossen *et al.*, 1987) that had been digested with *Sa* I (partially) and *Ns* I. The resulting plasmid was designated pGKV210-spc. pE194 (Horinouchi & Weisblum, 1982) was used as a template for the amplification of a promoterless erythromycin-resistance gene. To do this we used the primers 5'-GGGTCGACCCTATAACCAAATTAAAGAGGG-3' and 5'-CCCAAGCTTGGGCAGTTTATGCATCCCTTAAC-3' in a PCR reaction. These primers contained *Sal*I and *Hin*dIII restriction sites at their respective 5'-ends. The amplified fragment was digested with *Sal*I and *Hin*dIII and the fragment was ligated into pGKV210-spc that had been digested with *Sal*I and *Hin*dIII. The resulting plasmid was designated pIVS-E. To construct pIVS-PE the promoter region of the *mrp* gene was inserted into pIVS-E 5' to the promoterless erythromycin-resistance gene. The promoter region of the *mrp* gene was

amplified by PCR from pMRP11 (Smith *et al.*, 1992) using the primers 5'-CCCAAGCTTGGGAATTCATAATGTTTTTTTGAGG-3' and 5'-GCGTCGACATCTACGCATAAAAAATCCCCC-3'. These primers contained *Eco*RI and *Sal*I sites at their respective 5'-ends. Amplified DNA was digested with *Eco*RI and *Sa* I and the resulting fragment was ligated into *Eco*RI and *Sal*I-digested pIVS-E.

Construction of genomic *S. suis* libraries in pIVS-E. *Alu* I partial digests of *S. suis* serotype 2 strain 10 DNA were size fractionated (500- 1000 bp) on a 0.8% (w/v) agarose gel. The purified fragments were ligated to *Sma*I and calf intestinal phosphatase digested pIVS-E and the ligation mixtures were transformed to *E. coli* XL2-blue cells. Spectinomycin-resistant colonies were selected. Analysis of the transformants by PCR showed that more then 80% contained an insert. From 15 pools of about 2000-3000 independent *E. coli* transformants plasmid DNA was isolated. This plasmid DNA was subsequently used for the electrotransformation of *S. suis* strain 10 (Smith *et al.*, 1995). This resulted in approximately 30,000 independent *S. suis* transformants. The transformants were pooled and stored at -80 °C.

DNA techniques. Routine DNA manipulations and PCR reactions were performed as described by Sambrook *et al.* (1989). DNA sequences were determined on a 373A DNA Sequencing System (Applied Biosystems). Samples were prepared by using the ABI/PRISM dye terminator cycle sequencing ready reaction kit (Applied Biosystems). Custom-made sequencing primers were purchased from Life Technologies. Sequencing data were assembled and analyzed using the McMollyTetra software package. The BLAST program was used to search for protein sequences similar to the deduced amino acid sequences.

PCR reaction mixtures (50 µl) contained 10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 0.2 mM of each of the four deoxynucleotide triphosphates, 1 µM of each of the primers and 1 U of AmpliTaq Gold DNA polymerase (Perkin Elmer Applied Biosystems). DNA amplification was carried out in a Perkin Elmer 9600 thermal cycler and the program consisted of an incubation for 10 min at 95 °C and 30 cycles of 1 min at 95 °C, 2 min at 56 °C and 2 min at 72 °C.

Assessment of erythromycin levels in treated piglets. One-week-old specific pathogen free (SPF) piglets were treated orally with erythromycin stearate (Abbott, 20 or 40 mg body weight kg^{-1}) or intramuscularly with erythromycin (Erythrocin 200; Sanofi Santé, 20 or 40 mg body weight kg^{-1}). Blood samples were collected 3 hours, 6 hours or 24 hours after the administration of the antibiotics to determine erythromycin levels.

Experimental infections. Gnotobiotic Great Yorkshire and Dutch Landrace crossed piglets, were obtained from sows by caesarian section. The surgery was performed in sterile flexible film isolators. They were allotted to groups, each consisting of 4 piglets, and were housed in sterile stainless steel incubators. Housing conditions and feeding regimens were as described before (Vecht *et al.*, 1989; Vecht *et al.*, 1992). One week old piglets were inoculated intravenously with *S. suis* strain 10 (pIVS-E), 10 (pIVS-PE) or 10 (pIVS-RE) as described before (Vecht *et al.*, 1989; Vecht *et al.*, 1992, Table 3). Two hours after infection the pigs were injected intramuscularly with erythromycin for the first time and thereafter received erythromycin twice a day: once intramuscularly (Erythrocin, 40 mg body weight kg^{-1}) and once orally (erythromycin stearate, 40 mg body weight kg^{-1}). Piglets were monitored twice a day for clinical signs of disease, such as fever, nervous signs and lameness. Blood samples were collected three times a week from each pig. Leucocyte concentrations were determined using a conducting counter (Contraves A. G., Swizerland). To monitor infection with *S. suis* and to check for absence of contaminants, we collected swabs of the nasopharynx and of faeces daily. The swabs were plated directly onto Columbia agar containing 6% (v/v) horse blood. After the piglets were killed, they were examined for grosspathological changes. Tissue specimens were collected from the central nervous system, serosae, joints, lungs, heart and tonsils. The tissues were homogenized in the presence of Todd-Hewitt medium using an Ultra-Turrax tissuemizer (Omni International) and frozen at -80 ° C in the presence of 15% (v/v) glycerol.

RESULTS

Promoter selection system.

The plasmid pIVS-E was constructed to allow introduction of *S. suis* DNA fragments into a number of unique restriction sites in front of a promoterless erythromycin-resistance gene. The plasmid carries the origin of replication of pWVO1, which functions in *E. coli* as well as in *S. suis* (Smith *et al.*, 1995). *S. suis* strain 10 cells containing pIVS-E were sensitive to 1 μg erythromycin ml^{-1} on agar plates. In pIVS-PE the promoter of the *mrp* gene of *S. suis* (Smith *et al.*, 1992), which is highly expressed *in vivo* as well as *in vitro*, drives expression of the erythromycin-resistance gene. *S. suis* strain 10 cells containing pIVS-PE were resistant to high concentrations of erythromycin (> 256 μg erythromycin ml^{-1}) on agar plates. A *S. suis* DNA library in pIVS-E (pIVS-RE) was constructed and 30,000 individual *S. suis* clones or mutants were obtained. As determined by analysis of 24 randomly selected transformants, more than 80% of these clones or mutants contained an insert (results not shown). Moreover, 2% of the clones were resistant to 1 μg erythromycin ml^{-1} on agar plates, indicating the presence of some promoter sequences that were functional *in vitro*.

Selection of promoters induced under iron-restricted conditions.

We first selected for gene-sequences that were specifically induced on agar plates under iron-restricted conditions. For this purpose, about 96,000 c.f.u. were plated under iron-limiting conditions on agar plates containing deferoxamine mesylate and erythromycin. The 1500 colonies that grew on these plates were inoculated onto plates containing erythromycin, deferoxamine mesylate and $FeSO_4$. Twenty-four clones showed reduced growth in the presence of $FeSO_4$. The inserts of the 24 selected *iri* clones were amplified by PCR using primers complementary to the 5' ends of the erythromycin- and spectinomycin resistance genes and the nucleotide sequences of these fragments were determined. The sequence data showed that the 24 clones contained 18 unique sequences. The 18 sequences were analyzed for similarity to known genes by comparison with the sequences in the GenBank/EMBL and SWISSPROT databases. One sequence, *iri31*, was identical to *cps2A*, a previously identified *S. suis* gene putatively involved in the

regulation of capsule expression (Smith *et al.*, 1999). Fourteen *iri* sequences were similar to sequences of known, non-*S. suis*, genes. Three of these sequences (*iri2, iri1, 6* and *22,* and *iri34*) were similar to sequences of environmentally regulated genes previously selected by applying the IVET to *V. cholerae* (Camilli & Mekalanos, 1995), *S. aureus* (Lowe *et al.*, 1998) and *P. aeruginosa* (Wang *et al.*, 1996), respectively. One, contained in *iri1, 6,* and *22,* was similar to the *agrA* gene of *Staphylococcus aureus,* a key locus involved in the regulation of numerous virulence proteins. Three *iri* sequences had no significant similarity to any sequences in the databases (Table 2).

Conditions for selection of promoter sequences in piglets.

To determine the antibiotic treatment regime required for a successful selection of *in vivo* expressed promoter sequences, piglets were treated with different concentrations of erythromycin once a day. The erythromycin was administered either orally or intramuscularly. Levels of erythromycin in sera were determined 3, 6 or 24 hours after treatment over one week. High erythromycin levels were detected 3 h and 6 h after both treatments (results not shown). However, twenty-four hours after the treatments, the levels decreased dramatically. Based on these data we hypothesized that, for efficient promoter selection, it was necessary to treat the animals twice a day with erythromycin (40 mg kg^{-1}), once intramuscularly (at 9 a.m.) and once orally (at 4 p.m.).

To test this hypothesis we inoculated pigs either with *S. suis* strain 10 (pIVS-PE) or with strain 10 (pIVS-E). In pIVS-PE the promoter of the *mrp* gene of *S. suis* (Smith *et al.*, 1992), which is highly expressed *in vivo* as well as *in vitro,* drives expression of the erythromycin resistance gene. The control plasmid, pIVS-E, does not contain a promoter in front of the erythromycin resistance gene. The strains were inoculated intravenously or intranasally. All pigs infected with strain 10 (pIVS-PE) showed specific *S. suis* symptoms (Table 3) and, except for one, all pigs died in the course of the experiment. Moreover, high numbers of bacteria were isolated from the central nervous system, the serosae and from the joints. In contrast, none of the pigs inoculated with strain 10 (pIVS-E) showed specific clinical signs of disease and all survived the infection until the end of the experiment. Moreover, bacteria were not isolated from the central nervous

system, the serosae or from the joints of these animals. These data clearly demonstrated that *in vivo* expressed sequences could be selected from pigs using the applied antibiotic treatment regimen.

Selection of gene sequences expressed *in vivo* in piglets.

Piglets were inoculated intravenously with different doses (5×10^5 to 5×10^8 c.f.u.) of the *S. suis* library (Table 3) and treated with erythromycin as described above. Specific signs of disease developed in all animals, three to eight days after infection (Table 3). High numbers of bacteria were recovered from tissues (central nervous system, joints, serosae, lung, lever, spleen, heart and kidney) of the individual piglets. Analysis of the recovered bacteria showed that only a limited number of different clones were present in each of the bacterial samples isolated from the diseased pigs. For example, 30 randomly selected clones from the joints of one pig all possessed identical DNA inserts as assessed by PCR and DNA sequence analysis (results not shown). In addition, at 80% of the 62 sample sites analyzed, four randomly selected clones were all identical. However, from different tissues of a single animal, different clones or mutants could be isolated. On the other hand, identical clones could be isolated from different as well as from corresponding tissues of different animals. These findings indicated that a limited number of clones had been selected *in vivo* and were greatly enriched in the affected tissues. The observed selection was not tissue specific. Finally, none of the selected clones failed to grow on agar plates that contained 1 μg erythromycin ml^{-1}.

Two-hundred and forty five clones were analyzed by PCR and partial sequence analysis. Among these, 22 unique *ivs* clones were found. The 22 sequences were analyzed for similarity to sequences of known genes by comparison with the GenBank/EMBL and SWISSPROT databases (Table 4). The sequences of two genes showed similarity to genes encoding putative virulence factors: *ivs21, 26* and *30* which was identical to the *epf* gene, a previously identified *S. suis* gene, putatively involved in virulence (Smith *et al.*, 1993; Smith *et al.*, 1996); and *ivs31*, which was similar to the fibronectin-binding protein of *S. gordonii*. Moreover, the sequences of 2 *ivs* genes (*ivs25* and *ivs6, 7, 1*and *14*) were homologous to 2 environmentally regulated *ivi* genes, previously identified using

IVET selection in other bacterial species (Camilli & Mekalanos, 1995; Lowe *et al.*, 1998). Four *ivs* sequences (*ivs25; ivs23* and *24; ivs2, 4* and *28*; and *ivs6, 7, 13*and *14*) were also found when the library was selected using iron-restricted conditions. The remainder of the sequences showed similarity to sequences of known, non-*S. suis* genes, including 2 genes showing similarity to mobile elements and 5 genes showing similarity to genes of unknown function.

We describe the identification of environmentally regulated genes of *S. suis* serotype 2 by the use of iron-restricted conditions and by experimental infection of piglets. Eighteen unique *iri* genes and 22 unique *ivs* genes were found. None of the *ivs* genes was exclusively expressed *in vivo*. Four *iri* genes were identical to four clones selected *in vivo*. The selected gene sequences encode for potential virulence factors, expand our knowledge about the pathogenesis of *S. suis* infections in pigs and are of value in control of the disease either by the development of effective vaccines or by the development of new diagnostic methods. We used a promoter trap to identify environmentally regulated *S. suis* genes expressed under specific conditions, i.e. during iron-restriction or during experimental infection. This system differs from the antibiotic-based IVET system described for *S. typhimurium* (Mahan *et al.*, 1995) in two ways. One is that the *lacZ* reporter gene fusion is omitted in our vector constructions because inclusion of the *lacZ* gene resulted in structural instability of the vector. The other difference is that we used a plasmid system rather than a chromosomal integration system. We used a plasmid system because the low transformation efficiency of *S. suis* (Smith *et al.*, 1995) might prevent the generation of a complete gene library using a chromosomal integration system. From the data obtained, it is evident that we selected for a number of inducible and environmentally regulated sequences. Interestingly, four *iri* genes were identical to four *ivs* genes. Because most bacteria require iron for their growth and because there is a limited amount of free iron available within the host (Payne, 1993), it might be expected that the expression of some *ivs* genes is regulated by iron. With the *in vivo* selection system we did not observe tissue-specific colonization: clones isolated from one piglet were also isolated from other piglets from corresponding as well as from different tissues. This might be due to the mechanisms involved in the molecular pathogenesis of *S. suis* infections in pigs. Furthermore, it was striking, and different from the observations made with

IVET systems, that only a limited number of clones could be selected. In addition, we were not able to demonstrate that we selected for gene sequences that are exclusively expressed *in vivo*. This could be explained either by the absence of promoter sequences exclusively expressed *in vivo* among the 22 identified *ivs* genes, and/or by the inability of this plasmid-based system to identify such sequences due to gene dose effects.

A number of interesting genes were selected. Two *ivs* genes showed similarity to genes encoding putative virulence factors. *Ivs21, 26* and *30,* were shown to be identical to the *epf* gene of *S. suis* (Smith et al., 1993), which is found in virulent strains of *S. suis* serotypes 1 and 2 (Stockhofe-Zurwieden *et al.*, 1996; Vecht *et al.*, 1991; Vecht *et al.*, 1992). *Ivs31* showed similarity to the fibronectin/fibrinogen-binding protein of *S. gordonii* (accession no. X65164) and group A streptococci (Courtney *et al.*, 1994). In streptococci, fibronectin/fibrinogen-binding proteins play an important role in adhesion to host cells and are considered to be important virulence factors. The selection of these two *ivs* genes clearly demonstrated the selectivity of the system and might be indicative for the relevance of the other *ivs* genes in the pathogenesis of *S. suis* infections in pigs. The performance of the system was further demonstrated by the observation that two *ivs* genes, *ivs25* and *ivs6, 7, 13* and *14* showed similarity to environmentally regulated genes previously identified using an IVET selection system in other bacterial species.

Ivs25 showed significant similarity to the *sapR* gene of *S. mutants* (accession no. P72485) and *Lactobacillus sake* Lb706 (Axelsson & Holck, 1995) as well as to the *agrA* gene of *S. aureus* (Projan & Novick, 1997), both of which encode response regulator proteins of bacterial two-component signal-transduction systems, thereby mediating the response to an environmental signal (Projan & Novick, 1997). Use of an IVET selection system for *S. aureus* in mice selected the region preceding the *agrA* gene, suggesting induction of *agrA* expression under *in vivo* conditions (Lowe *et al.*, 1998). Moreover, in *S. aureus* the *agr* locus was shown to play an important role in altering the expression of a considerable number of virulence factors in response to cell density (Projan & Novick, 1997). In future experiments the putative role of *ivs25* in the expression of virulence factors in *S. suis* will be analyzed further.

Clones *ivs6, 7, 13* and *14* showed similarity to a gene, *iviVI*, previously identified by IVET selection in *V. cholerae* (Camilli & Mekalanos, 1995). The function of *iviVI* is unknown. However, the genes showed similarity to members of the ATP-binding cassette family of transporters. The sequenced portion of *ivs6, 7, 13* and *14* included an N-terminal ATP-binding Walker A box motif, which is highly conserved in this transporter family.

Four *ivs* genes were identical to four *iri* genes. The first gene, *ivs23* and *24,* which is identical to *iri24,* showed similarity to *cpsY* of *S. agalactiae* (Koskiniemi *et al.*, 1998) and to *oxyR* of various organisms (Demple, 1999). CpsY of *S. agalactiae* is involved in the regulation of capsule expression and environmental induction of expression of the *cpsY* gene has been suggested by Koskiniemi *et al.* (1998). In *S. suis ivs23* and *24* and *iri24* are not linked to the capsular locus (Smith *et al.*, 1999). The *oxyR* gene is the central regulator of oxidative stress response in *E. coli* (Demple, 1999) and approximately 10 genes are under the control of the OxyR protein. The second gene, *ivs2, 4* and *28,* which is identical to *iri10* and *20,* showed similarity to the *yoaE* gene of *E. coli* (accession no. P76262), a putative ABC transporter protein. The third and the fourth genes, *ivs25* and *ivs6, 7, 13* and *14* were identical to *iri1, 6* and *22* and *iri2,* respectively. These genes also showed similarity to *ivi* genes selected using IVET in other bacterial species.

Based on data, recently presented by Niven *et al.* (1999), selection of *iri* genes of *S. suis* is not expected. The authors described that *S. suis* does not require iron for growth. However, in their studies the authors used media reduced from iron by using ethylenediamine di-o-hydroxyphenylacetic acid (EDDA). Therefore, the different conditions used in vitro may explain the different results obtained.

Two of the *S. suis ivs* genes, *ivs1* and *ivs8,* showed similarity to transposon sequences. Moreover, one *S. suis ivs* gene, *ivs2, 4* and *28,* had a GC% that was considerably higher than the composition of the rest of the selected genes. The relevance of these *ivs* genes inr the pathogenesis of *S. suis* infections in pigs needs to be investigated further. However, it is striking that in *S. typhimurium* several of the *ivi* clones, that are required for full virulence have been found to be associated with mobile elements. Their atypical base composition and codon

usage has led to the suggestion that they have been acquired from other bacterial species by horizontal transfer (Conner *et al.*, 1998).

Importantly, our screen identified 5 *ivs* genes that showed similarity to sequences encoding proteins of unknown function. These genes are not standard housekeeping or metabolic genes. Therefore, strains of *S. suis* carrying mutations in each of these genes are currently being constructed and the effect of these mutations on bacterial virulence are examined.

Besides the four *ivs/iri* genes, a considerable number of other *iri* genes have been selected in this study by plating the library under iron-restricted conditions. Interestingly, one of the selected *iri* genes, *iri31*, is identical to the *cps2A* gene of *S. suis*. This gene was previously isolated as a part of the capsular locus of *S. suis* serotype 2 (Smith *et al.*, 1999) and was implicated in the regulation of capsular polysaccharide biosynthesis (Kolkman *et al.*, 1997; Smith *et al.*, 1999). Moreover, because the capsule of *S. suis* is expressed in larger size after *in vivo* growth when compared to growth *in vitro* (Quessy *et al.*, 1994), regulated expression of *cps2A* might be expected. Another *iri* gene, *iri7*, showed similarity to the *rpgG* gene of *S. mutans*. This gene was shown to be required for the biosynthesis of rhamnose-glucose polysaccharide (Yamashita *et al.*, 1999). Because rhamnose is part of the polysaccharide capsule in *S. suis* serotype 2 (Elliott & Tai, 1978), a role of the *iri7* gene in capsule biosynthesis can be proposed. *Iri34* showed similarity to the *np16* gene, previously identified using IVET selection in *P. aeruginosa* and suspected to encode threonine dehydratase activity (Wang *et al.*, 1996). Together with the observation that 4 *iri* genes could be selected by the *in vivo* approach, these data show that the *iri* genes encode important virulence factors for *S. suis*.

Further example

Contribution of Fibronectin-Binding Protein to Pathogenesis of *Streptococcus suis* serotype 2

Streptococcus suis causes severe infections in piglets. The bacterial infections include meningitis, septicemia, and arthritis and the animals often do not survive the infection (6, 28). Occasionally, *S. suis* causes septicemia and meningitis in humans (3). The pathogenesis of an *S. suis* infection is rarely understood. Sows are symptomless carriers of *S. suis* on their tonsils, and pass the bacteria on to their piglets. The piglets cannot cope with the bacterium and subsequently develop the specific symptoms of an *S. suis* infection. Until now, 35 capsular serotypes of *S. suis* have been described (26), but serotype 2 strains are most often isolated from diseased piglets. Capsule is an important virulence factor, since piglets infected with an acapsular mutant of *S. suis* serotype 2 strains do not develop any clinical symptoms (22). Bacterial proteins have been suggested to play a role in the pathogenesis as well (2, 26). The expression of murimidase-released protein (MRP) , extracellular factor (EF) and suilysin was shown to be strongly associated with pathogenic strains of *S. suis* serotype 2 (1, 29, 30). Since isogenic mutants lacking MRP and EF, and isogenic mutants lacking suilysin were still pathogenic for young piglets, these proteins are not absolutely required for virulence (2, 23). Recently, a new virulence factor was identified (21) by using a complementation approach. The function of this virulence factor in the pathogenesis has to be further investigated.

Many important virulence factors are environmentally regulated and are induced at specific stages of the infection process (15). To identify these genes in *S. suis,* we cloned promoters and their downstream sequences that are "on" during experimental *S. suis* infection of piglets (20). Twenty-two *in vivo* selected (*ivs*) genes were found. Two of the *ivs* genes were directly linked to virulence since homology was found to genes in the database that encode for known virulence factors. One of these *ivs* genes (*ivs-21*) was identical to the *epf* gene of

virulent *S. suis* serotype 2 strains (30). The other (*ivs-31*) showed homology to genes encoding fibronectin-/fibrinogen-binding proteins of *Streptococcus gordonii* (GenBank accession no. X65164) and *Streptococcus pyogenes* FBP54 (8). A considerable number of fibronectin-binding proteins of various bacterial species have been shown to be important virulence factors (12). In *S. pyogenes*, FBP54 was shown to be expressed in the human host and to preferentially mediate adherence to human buccal epithelial cells (7). It was recently shown that the FBP54 protein induces protective immunity against *S. pyogenes* challenge in mice (13).

In the present study we describe a fibronectin-/fibrinogen-binding protein of *S. suis* (FBPS). The sequence of *fbps* was determined. Binding studies showed that purified FBPS bound fibronectin and fibrinogen. A contribution of FBPS to the pathogenesis of *S. suis* serotype 2 was found.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 5. *S. suis* strains were grown in Todd-Hewitt broth (code CM 189; Oxoid, Ltd.) and plated on Columbia blood base agar plates (code CM331; Oxoid, Ltd., London, United Kingdom), containing 6% (vol/vol) horse blood. *E. coli* strains were grown in Luria Broth (17) and plated on Luria Broth containing 1.5 % (wt/vol) agar. If required, antibiotics were added in the following concentrations: 50 :g/ml of spectinomycin (Sigma, St. Louis, Mo.) for *E. coli* and 100 :g/ml for *S. suis,* 100 :g/ml of ampicillin (Boehringer, Mannheim, Germany) for *E. coli* and 25 :g/ml of kanamycin (Boehringer) for *E. coli.*

DNA techniques and sequence analysis. Routine DNA manipulations were performed as described by Sambrook *et al.* (19). DNA sequences were determined on a 373A DNA Sequencing System (Applied Biosystems, Warrington, Great Britain). Samples were prepared by use of an ABI Prism dye terminator cycle sequencing ready reaction kit (Applied Biosystems). Sequencing data were assembled and analyzed using the Lasergene program (DNASTAR). The BLAST software package was used to search for protein sequences homologous to the deduced amino acid sequences in the GenBank/EMBL databases.

Southern blotting and hybridization. Chromosomal DNA was isolated as described by Sambrook *et al.* (19). DNA fragments were separated on 0.8% agarose gels and transferred to GeneScreen Plus hybridization transfer membrane (NEN™ Life Science Products, Boston, USA) as described by Sambrook *et al.* (19). DNA probes of the *fbps* and *spc* genes were labeled with [α-^{32}P]dCTP (3,000 Ci/mmol; Amersham Life Science, Buckinghamshire, Great

Britain) by use of a random primed DNA labeling kit (Boehringer). The DNA on the blots was pre-hybridized for at least 30 min at 65°C and subsequently hybridized for 16 h at 65°C with the appropriate DNA probes in a buffer containing 0.5 M sodium phosphate [pH 7.2], 1 mM EDTA and 7% sodium dodecyl sulphate. After hybridization, the membranes were washed twice with a buffer containing 40 mM sodium phosphate [pH 7.2], 1 mM EDTA and 5% sodium dodecyl sulphate for 30 min at 65°C and twice with a buffer containing 40 mM sodium phosphate [pH 7.2], 1 mM EDTA and 1% sodium dodecyl sulphate for 30 min at 65°C. The signal was detected on a phosphor-imager (Storm; Molecular Dynamics, Sunnyvale, Calif.).

Construction of a *fbps* knock-out mutant. To construct the mutant strain 10)FBPS, the pathogenic strain 10 (27, 29) of *S. suis* serotype 2 was electrotransformed (24) with the plasmid pFBPS7-47. In this plasmid, the *fbps* gene was inactivated by the insertion of a spectinomycin resistance gene. To create pFBPS7-47 (Fig. 1) the 382 bp *Sal*I-*Sal*I fragment of pFBPS7-46 was replaced by the 1.2 kb *Eco*RV-*Sma*I fragment of pIC-Spc, containing the spectinomycin resistance gene, after the *Sal*I sites of the vector were made blunt (Fig. 1). After electrotransformation of strain 10 with pFBPS7-47, spectinomycin resistant colonies were selected on Columbia agar plates containing 100 :g/ml of spectinomycin. Southern blotting and hybridization experiments were used to select for double crossover integration events (data not shown).

FBPS expression construct. To construct an FBPS expression plasmid the QIA*express* Kit (Qiagen GmbH, Hilden, Germany) was used. The primers corresponded to positions 250 to 272 and from 1911 to 1892 of the *fbps* gene. The sequences of these primers were 5'(GCGGATCCGATGACGATGACAAATCTTTTGACGGATTTTTTTTTAC)3' and

5'(CCCAAGCTTGGGCATGAACTAGATTTTCATGG)3'. The primers contained restriction sites for *Bam*HI and *Hin*dIII respectively to amplify the *fbps* gene from pFBPS7-47. The amplified PCR product was digested with *Bam*HI and *Hin*dIII and the 1.8 kb *fbps* gene was cloned into pQE-30 digested with *Bam*HI and *Hin*dIII, yielding pQE-30-FBPS. pQE-30-FBPS was transformed to M15[pREP4].

Purification of FBPS. M15[pREP4][pQE-30-FBPS] was used to express and to purify the FBPS using the QIA*expressionist*™ (Qiagen). In short, M15[pREP4][pQE-30-FBPS] cells were grown exponentially; 1 mM IPTG was added and the cells were allowed to grow another 4 hr at 37°C. Subsequently, cells were harvested and lysed. The cleared supernatants were loaded onto Ni^{2+}-NTA agarose columns. FBPS containing a 6 x HIS tag was bound to the Ni^{2+}-column. The columns were washed and the protein was eluted. Different buffers were used for native and for denaturing purification. FBPS purified under denaturing conditions was renaturated on a Ni^{2+}-NTA column by using a linear 6 M - 1 M urea gradient in 500 mM NaCl, 20% glycerol and 20 mM Tris-HCl [pH7.4], containing protease inhibitors (25 :g/ml of pefabloc, 0.7 :g/ml of pepstatin, 1 :g/ml of aprotinin, 0.5 :g/ml of leupeptin) . All procedures were performed according to the manufacturer's recommendations. The 6 x HIS tag was removed from the protein by incubating purified FBPS in 20 mM Tris-HCl [pH 7.4], 50 mM NaCl, 2 mM $CaCl_2$ and 0.5 U of light chain enterokinase (New England Biolabs, Beverly, Mass.) for 16 h at RT.

Immunization of rabbits with FBPS. Purified and renaturated FBPS was used to immunize 2 rabbits. To remove urea the protein was dialyzed against phosphate buffered saline (136 mM NaCl; 2.68 mM KCl; 8.1 mM Na_2HPO_4; 2.79 mM KH_2PO_4 [pH7.2]) over night at 4°C. Seven days before

immunization blood was collected from the rabbits to determine the natural titers against FBPS. At day 1 those rabbits with negative anti-FBPS titers, were immunized intramuscularly with 2 times 0.5 ml of 100 :g/ml of FBPS in a water-in-oil emulsion (Specol; ID-Lelystad). At day 28, rabbits were immunized for the second time using the same amount of protein and the same route of immunization. Three weeks after the second immunization the rabbits were sacrificed and blood was collected. The blood was coagulated and serum was collected and used for immunodetection of FBPS.

Immunodetection of FBPS. Proteins were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) by standard procedures (19). Proteins in the gel were visualized using SYPRO-orange (Molecular Probes, Sunnyvale, Calif.) staining according to the manufacturer's recommendations. Signals were detected on a phosphor-imager (Storm; Molecular Dynamics). A known bovine serum albumin concentration range was used as a standard, to calculate the amounts of protein present in the gel. The Molecular Dynamics program was used for the calculations.

Proteins were transferred to a nitrocellulose membrane by standard procedures (19). The membranes were blocked in Blotto: Tris-buffered saline (TBS) (50 mM Tris-HCl [pH 7.5], 150 mM NaCl) containing 4% skimmed milk and 0.05% Tween 20, at room temperature (RT) for 1 h. To detect recombinant purified FBPS, membranes were incubated with a monoclonal antibody against the 6 x HIS tag (Clontech, Palo Alto, CA.) in a 1:10,000 dilution in Blotto-TBS (1:1) at RT for 1 hr, followed by an incubation with alkaline phosphatase-conjugated anti-mouse antibody in a 1:1,000 dilution in Blotto-TBS (1:1) at RT for 1 hr. Reactivity of purified FBPS was tested by using a convalescent serum of a pig that had survived an *S. suis* infection. Nitrocellulose membranes were

incubated with the polyclonal pig serum in a 1:200 dilution in Blotto-TBS (1:1) at RT for 1 hr, followed by an incubation at RT for 1 h with alkaline phosphatase-conjugated anti-swine antibody in a 1:2,000 dilution in Blotto-TBS (1:1). As a substrate Nitro Blue Tetrazolium (Merck, Darmstadt, Germany)-bromochloroindolyl phosphate (Sigma) was used. All washing steps were performed in Blotto-TBS (1:1).

Fibronectin- and fibrinogen binding. Binding studies were performed by indirect Western blotting. Proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane as described above. The membranes were blocked in MPBS: PBS containing 4% skimmed milk and 0.05% Tween 20. Subsequently, the membrane was incubated with 5 :g/ml of human fibronectin (fn) (Sigma) or 5 :g/ml of human fibrinogen (fgn) (Sigma) in PBS containing 5% fetal calf serum, 2% NaCl, and 0.05% Tween 80 at RT for 1 hr. To detect bound fibronectin and fibrinogen, the membranes were incubated with horse-radish peroxidase-conjugated anti-fibronectin (DAKO) or anti-fibrinogen (DAKO) antibodies in a 1:1,000 dilution in PBS containing 5% fetal calf serum, 2% NaCl, and 0.05% Tween 80 at RT for 1 hr. The signal was visualized by using ECL$^+$ (Amersham Pharmacia Biotech, N. J.) according to the manufacturer's recommendations. Signals were detected on a phosphor-imager (Storm; Molecular Dynamics). All washing steps were performed in MPBS-PBS (1:1).

Experimental infections. Germfree piglets, crossbreeds of Great Yorkshire and Dutch Landrace, were obtained from sows by cesarean sections. The surgery was performed in sterile flexible film isolators. Piglets were allotted to groups of 4, and were housed in sterile stainless steel incubators. Housing conditions and feeding regimens were as described before (27, 29). Six-day-old piglets were inoculated intranasally with about 10^7 cfu of *Bordetella*

bronchiseptica 92932, to predispose the piglets to infection with *S. suis*. Two days later they were inoculated intranasally with 10^6 cfu of *S. suis* strain 10 plus 10^6 cfu of *S. suis* strain 10)FBPS. To determine differences in virulence between wild-type and mutant strains, LD_{50} values should be determined. To do this, large numbers of piglets are required. For ethical reasons this is not acceptable. To circumvent this problem we decided to perform co-colonization studies. To monitor for the presence of *S. suis* and *B. bronchiseptica* and to check for absence of contaminants, swabs taken from the nasopharynx and the feces were cultured three times a week. The swabs were plated directly onto Columbia agar containing 6% horse blood, or grown for 48 hr in Todd-Hewitt broth and subsequently plated onto Columbia agar containing 6% horse blood. Pigs were monitored twice a day for clinical signs and symptoms, such as fever, nervous signs, and lameness. Blood samples from each pig were collected three times a week. Leukocytes were counted with a cell counter. The piglets were killed when specific signs of an *S. suis* infection were observed, such as arthritis or meningitis, or when the pigs became mortally ill. The other piglets were killed 2 weeks after inoculation with *S. suis* and examined the same way as the piglets that were killed based on their clinical symptoms. All piglets were examined for pathological changes. Tissue specimens from heart, lung, liver, kidney, spleen, and tonsil, and from the organs specifically involved in an *S. suis* infection (central nervous system (CNS), serosae, and joints) were sliced with a scalpel or a tissuenizer. Tissue slices from each organ or site were resuspended in 2 - 25 ml of Todd-Hewitt containing 15% glycerol, depending on the size of the tissue slice. The suspension was centrifuged at 3,000 rpm for 5 min. The supernatant was collected and serial dilutions were plated on Columbia agar containing 6% horse blood, as well as on Columbia agar plates containing 6% horse blood and 100

:g/ml of spectinomycin to quantitate the number of wild type and mutant bacteria present. The number of mutant strain 10)FBPS cells was determined by counting the number of CFU on the appropriate serial dilution on the selective plates; the number of wild-type strain 10 cells was determined by counting the number of CFU on the appropriate serial dilution on the Columbia Agar blood plates of which the number of CFU counted on the selective plates was subtracted. When wild type and mutant bacteria were found in tissues, the ratio of wild-type and mutant strain was determined again, by toothpicking about 100 individual colonies onto both Columbia Agar plates and onto Columbia Agar plates containing 100 :g/ml spectinomycin .

All animal experiments were approved by the ethical committee of the Institute for Animal Science and Health in accordance with the Dutch law on animal experiments.

Nucleotide sequence accession number. The nucleotide sequence data of *fbps* have been submitted to GenBank, in which the sequence is listed under accession no. AF438158.

RESULTS

Cloning of the *S. suis fbps* gene. One of the *in vivo* selected genes (*ivs*-31) (20), showed homology to the 5' part of genes encoding for FlpA and FBP54, fibronectin binding proteins (FnBP) of *Streptococcus gordonii* (GenBank accession no. X65164) and *Streptococcus pyogenes* (8), respectively. To clone the entire *fbps* gene of *S. suis*, *ivs*31 was subsequently used as a probe to identify a chromosomal DNA fragment of *S. suis* serotype 2 containing flanking *fbps* sequences. A 5 kb *Eco*RI fragment was identified and cloned in pGEM7Zf(+) yielding pFBPS7-46 (Fig. 1). Sequence analysis revealed that this fragment contained the entire *fbps* gene of *S. suis* serotype 2. An open reading frame of 1659 bp coding for a polypeptide of 553 amino acids was found. The putative ATG start codon is preceded by a sequence similar to ribosome binding sites of gram-positive bacteria. Further upstream, two putative promoter sequences could be identified. Upstream of these promoter sequences of *fbps* a direct repeat was found that could serve as a transcription terminator of the gene located 5' of *fbps*. Downstream of *fbps* a gene was found that showed homology to an alpha-acetolactate decarboxylase was found. This gene is transcribed in the opposite direction of *fbps*. The deduced amino acid sequence was aligned with that of several previously identified FnBPs from other bacteria. As expected, FBPS was very homologous to FlpA of *S. gordonii* (76%) and also showed homology to FnBP's of other organisms, like *Streptococcus pneumoniae* (73%), *S. pyogenes* (69%), *Lactococcus lactis* (59%), and *Bacillus subtilis* (41%). Compared to the sequence of FBP54, FBPS has a longer N-terminus with 76 additional amino acids. This longer N-terminus was also seen in other organisms like *S. gordonii*, *S. pneumoniae* and *B. subtilis*. In FBP54 the primary fibronectin-/fibrinogen-

binding domain was localized to its N-terminal part, to the first 89 amino acids (8). Over this region the homology of FBPS to FBP54 is very high (80%) suggesting that FBPS can bind both fibronectin and fibrinogen.

Binding of FBPS to fibronectin and fibrinogen. To confirm the binding of FBPS of *S. suis* to fibronectin (fn) and fibrinogen (fgn), FBPS was purified under native conditions. A protein expression construct, which expresses FBPS with a 6 x HIS tag fused to the N-terminus, was used for this purification. Four hundred :g of FBPS was purified from 50 ml of exponential-phase *E. coli* cells after induction with IPTG. The purity of this FPBS was determined with SDS-PAGE and Western blotting (Fig. 2). The induced *E. coli* lysate contained a broad range of proteins, among which the 64 kDa protein FBPS was very clearly present (panel A, lane 1). After purification, highly purified FBPS with 6 x HIS tag was obtained (panel A, lane 2). When both samples were incubated with a monoclonal antibody against the 6 x HIS tag, FBPS was the only protein that was detected (panel B).

To determine whether FBPS binds fn and fgn, a Western blot containing purified FBPS was incubated with soluble human fn and human fgn (Fig. 3, panels A and B). Specific binding of fn and fgn to FBPS was clearly detected. No binding of fn and fgn to BSA, a negative control protein, was observed. To exclude possible background signals due to immunoglobulin-binding of FBPS, the same experiment was performed without addition of fibronectin or fibrinogen. No binding was found (Fig. 3, panels C and D), indicating that the binding was specific for fibronectin and fibrinogen. To control whether the binding of fn and fgn to FBPS, was not mediated by the 6 x HIS tag, the tag was removed by an enterokinase treatment. Figure 3, panels E and F clearly show that FBPS

without the 6 x HIS tag, still efficiently bound to fn and fgn. Therefore, it was concluded that FBPS can specifically bind to fn and fgn.

Immunogenicity of FBPS. Since it was shown that FBP54 induced a protective immune response in mice against a lethal dose of *S. pyogenes* (13), we next determined whether purified FBPS was recognized by convalescent serum of a pig that survived an *S. suis* infection. As shown in figure 2 panel C, the FBPS clearly reacted with this anti-serum. When the same experiment was performed with non-immune serum of an SPF piglet, no band of the size of FBPS was detected (data not shown). These findings indicate that FBPS is expressed *in vivo* and that the protein is indeed immunogenic in young pigs.

Distribution of the *fbps* gene among the 35 *S. suis* serotypes. Since we were interested in a cross-protective vaccine candidate, we next analyzed the presence of the *fbps* gene among the various *S. suis* serotypes. *Ivs-31*, the clone containing the promoter and the 5'-part of the *fbps* gene was radiolabeled, and chromosomal DNA of the reference strains of the 35 different *S. suis* serotypes was hybridized with this probe. The 3 different phenotypes of *S. suis* serotype 2, a pathogenic, a non-pathogenic and a weak pathogenic strain, were included in this study as well. The *fbps* gene was present in all *S. suis* serotypes and phenotypes, except for serotypes 32 and 34 (Fig. 4).

Role of FBPS in pathogenesis. To test the role of FBPS in the pathogenesis of *S. suis*, an isogenic knock-out mutant of FBPS was constructed in strain 10, strain 10)FBPS. Since upstream of *fbps*, a direct repeat was found, that could serve as a transcription terminator, and downstream of *fbps* a gene showing homology to an alpha-acetolactate decarboxylase was found, that is transcribed in the opposite direction, polar effects to genes upstream or downstream of *fbps* are not expected. To verify that the mutant strain 10)FBPS

did not produce FBPS, protoplasts of strain 10 and strain 10)FBPS were subjected to SDS-PAGE and Western blotting. FBPS was detected using a polyclonal antiserum raised against purified FBPS. It was shown that strain 10)FBPS expressed no FBPS, while strain 10 did (data not shown). Subsequently the virulence of this mutant strain was tested in an experimental infection in piglets. The mutant strain 10)FBPS was used in a competition challenge experiment with the wild- type strain to determine the relative attenuation of the mutant strain. Using *in vitro* conditions, the growth rates of the wild-type and mutant strain in Todd Hewitt medium, were found to be essentially identical (data not shown). Wild-type and mutant strain were inoculated at an actual ratio of 0.65 (1.63^{e}6 cfu of wild-type bacteria ml^{-1} and 3.09^{e}6 cfu of mutant bacteria ml^{-1}). During the experiment, piglets that developed specific *S. suis* symptoms (meningitis, arthritis, or mortally illness) were killed. Piglets that did not develop these symptoms were killed at the end of the experiment. From all piglets the ratio of wild-type and mutant strain in various organs was determined. As shown in figure 5, panel A, similar numbers of wild-type and mutant bacteria were re-isolated from tonsils. The ratio was similar to the input ratio (ratio varied from 0.33 - 0.85, average 0.61). This clearly indicates that the efficiency of colonization of wild-type and mutant strain on tonsils was essentially identical. Apparently, FBPS is not strictly required for colonization of the tonsils of the piglets. Three out of four piglets developed clinical signs specific for an *S. suis* infection. Two piglets (4664 and 4666) showed clinical signs of arthritis, and one piglet (4668) showed clear central nervous signs. The fourth piglet did not develop any clinical signs. These observations coincided with pathomorphological abnormalities of the specific organs of an *S. suis* infection in post-mortem sections. As shown in figure 5, panel B, exclusively wild-type bacteria were re-isolated from the joints of piglet

4664, and from the CNS of piglet 4668. The numbers of CFU of wild type bacteria that were re-isolated from these specific organs were very high, while absolutely no mutant bacteria were found. From the joints of pig 4666 low numbers of both wild-type and mutant bacteria were re-isolated in a ratio of 0.84 (1.0e2 CFU of wild type bacteria and 5.2e2 CFU of mutant bacteria), a ratio essentially identical to the input ratio (Fig. 5, panel B). Southern blot experiments, using the *fbps* and the *spc* genes as probes, confirmed that the mutant bacteria isolated from the joint of pig 4666 were indeed identical to the input mutant bacteria. Taken together, these data indicate, that the FBPS mutant is capable of reaching and colonizing the specific *S. suis* organs (at least the joints), but that the mutant is far less efficiently recovered from organs than the wild type.

Table 1. Bacterial strains and plasmids

strain/plasmid	relevant characteristics*	source/reference
Strain		
E. coli		
XL2 blue		Stratagene
S. suis		
10	virulent serotype 2 strain	(Vecht *et al.*, 1992)
Plasmid		
pKUN19-spc	replication functions pUC, Amp^R, Spc^R	(Konings *et al.*, 1987, Smith *et al.*, 1995)
pGKV210	replication functions pWVO1, Cm^R, Em^R	(Van der Vossen *et al.*, 1987)
pE194	Em^R	(Horinouchi & Weisblum, 1987)
pMR11	pKUN19 containing *S. suis mrp* gene	(Smith *et al.*, 1992)
pIVS-E	replication functions pWVO1, Spc^R, promoterless *emR* gene of pE194	this work

pIVS-PE	pIVS-E containing promoter of *mrp* gene preceding the promoterless *emR* gene	this work
pIVS-RE	pIVS-E containing random *S. suis* sequences preceding the promoterless *emR* gene	this work

*

Spc^R: spectinomycin resistant

Amp^R: ampicillin resistant

Em^R: erythromycin resistant

Cm^R: chloreamphenicol resistant

TABLE 2. Iron-restriction induced *S. suis* genes

Clone	Insert (bp)	GC%	Data base homology (accession no)	Function of homologue	% Identity
			Regulatory functions		
iri 1, 6, 22	800	34	*S. mutans* SapR (U75483)	response regulator protein	44
			S. aureus AgrA (X52543)	response regulator protein	51
			S. aureus Ivi2		
iri 24	850	38	*S. agalactiae* CpsY (CAB36982)	regulation capsule expression	46
			E. coli OxyR (P11721)	oxidative stress regulator	51
iri 23	1000	38	*B. subtilis* YvyD (P28368)	sigma-54 modulator homologue	44
			Metabolic functions		
iri 7	800	39	*S. mutans* RgpG (Q9XDW8)	rhamnose-glucose biosynthesis	76
iri 11	700	34	*L. lactis* NrdD (Q9ZAX6)	anaerobic ribonucleotide reductase	51
iri 14	500	38	*S. pneumoniae* SulB (Q54614)	dihydrofolate synthetase	41
iri 16	850	48	*B. subtilis* TrmU (O35020)	RNA methyltransferase	62
iri 32	300	41	*C. histolyticum* RuvB (O9ZNJ5)	hypoxanthine-guanine phosphoribosyl transferase	55

	iri 34	1000	44	*L. lactis* IlvA (U92974) *P. aeruginosa* Pn16	probable threonine dehydratase	56
				Transporter functions		
	iri 2	750	36	*B. subtilis* YloD (O34328)	putative guanylate kinase	50
				S. gordonii ComYA (U81957)	putative ABC transporter	37
				Vibrio cholerae IviVI (Q56605)	putative ABC transporter	47
	iri 10, 20	1350	51	*E. coli* YoaE (P76262)	putative transport protein	94
				Unknown		
	iri 13, 15, 27	800	34	*M. tuberculosis* MTCY336_33 hypothetical protein (O06593)	unknown	38
	iri 29	850	36	*S. aureus* Yp15 (P13977) hypothetical protein	unknown	39
	iri 18	800	39	*S. crista* hypothetical protein (AAF61316)	unknown	82
	iri 3	700	36	no homology found		
	iri 4	700	36	no homology found		
	iri 8, 26	900	35	no homology found		

TABLE 3. Virulence of *S. suis* 10 (pIVET-E), 10 (pIVET-PE) and 10 (pIVET-RE) in gnotobiotic piglets

Strains/ library	No. of piglets	Dose (route of infection)	Mortality* [%]	Morbidity¥ [%]	Clinical index of the group		Fever index	Leuco-cyte index	No. of pigs from which *S. suis* was isolated		
					Specific	Non-specific			CNS	Serosae	Joints
10 (pIVS-E)	4	10^6 (i.n.)	0	0	0	6	9	75	0	0	0
10 (pIVS-E)	4	10^6 (i.v.)	0	0	6	12	31	0	0	0	0
10 (pIVS-PE)	4	10^6 (i.n.)	100	100	30	40	35	100	3	0	2
10 (pIVS-PE)	4	10^6 (i.v.)	75	100	50	42	43	50	3	3	4
10 (pIVS-RE)	4	5×10^5 (i.v.)	100	100	56	75	44	83	2	2	4
10 (pIVS-RE)	4	5×10^6 (i.v.)	100	100	43	73	43	60	3	0	4
10 (pIVS-RE)	4	5×10^7 (i.v.)	100	100	60	74	48	75	4	1	4
10 (pIVS-RE)	4	5×10^8 (i.v.)	100	100	49	70	37	50	3	3	4

* Percentage of pigs that died due to infection or had to be killed for animal welfare reasons

¥ Percentage of pigs with specific symptoms

¦ Percentage of observations which matched the described criteria

‖ Ataxia, lameness of at least one joint and/or stiffness

¤ Inappetance and/or depression

à Percentage of observations for the experimental group of a body temperature of > 400 C

£ Percentage of blood samples for the group in which the concentration of granulocytes was > 1010/liter

TABLE 4. ***S. suis*** **genes selected in pigs**

Clone	Sites of isolation	Insert (bp)	GC%	Data base homology (accession no)	Function of homologue	% Identity
				Putative virulence factors		
ivs 31	CNS	200	47	*S. gordonii* FlpA (X65164)	fibronectin/fibrinogen binding	70
				Regulatory functions		
ivs 25	joint	800	34	*S. mutans* SapR (P72485)	response regulator protein	49
				S. aureus AgrA (X52543)	response regulator protein	51
				S. suis Iri 1, 6, 22		100
ivs 23, 24	other	850	38	*S. agalactiae* CpsY (CAB36982)	regulation capsule expression	46
				E. coli OxyR (P11721)	oxydative stress regulator	51
				S. suis Iri 24		100
ivs 16	CNS	800	43	*S. epidermidis* AltR (U71377)	putative transcriptional regulator	26
ivs 20	lung	800	41	*L. lactis* AldR (O34133)	putative regulator AldR	64
				Metabolic functions		
ivs 33	CNS	570	36	*E. coli* ThrC (P00934)	threonine synthase	41

	ivs 5, 10, 12, 22	CNS, joint	900	36	*S. gordonii* Tdk (P47848)	thymidine kinase	87
	ivs 18	lung	730	32	*S. mutans* NADH oxidase (JC4541)	NADH oxidase	80
					Transporter functions		
5	ivs 2, 4, 28	CNS, joint	1350	51	*E. coli* YoaE (P76262)	putative transport protein	94
					S. suis Iri 10, 20		100
	ivs 3	joint	1000	42	*S.mutans* OrfU (AF267498)	putative ABC transporter (permease)	33
	ivs 6, 7, 13, 14	CNS, joint	1350	36	*B. subtilis* Ylo D (O34328)	putative guanylate kinase	50
					S. gordonii ComYA (U81957)	putative ABC transporter	37
10					*V. cholera* IviVI (Q56605)	putative ABC transporter	47
					S. suis Iri 2		100
					Transposases		
	ivs 8	CNS	600	41	*S. pneumoniae* transposase	transposase	70
15					(Z86112)		
	ivs 1	joint	1600	39	*C. perfringens* (X71844)	putative transposase	56
					Miscellaneous		
	ivs 32, 35	CNS		500	38 *S.typhimurium* FliF (P15928)	flagellar M-protein precursor	36
20	ivs 9, 17	joint, CNS	800	36	*B. subtilis* ComE ORF2 (P32393)	competence development	37
	ivs 11	serosea	800	44	*P. syringae* TabA (P31851)	diaminopimelate decarboxylase/ tabtoxin	53

					Unknown		
	ivs 15	CNS	750	42	*B. subtilis* conserved hypothetical protein YdiB (D88802)	unknown	43
	ivs 29	joint	800	38	*S.salivarius* hypothetical protein (AF130465)	unknown	79
	ivs 34	CNS	600	43	*B. subtilis* conserved hypothetical protein YrrK (O34634)	unknown	61
	ivs 36	joint	830	42	*B. subtilis* hypothetical protein YqeG (P54452)	unknown	35
	ivs 19	lung	950	34	*S.cristatus* hypothetical protein (U96166)	unknown	86

Table 5. Bacterial strains and plasmids

Strain/plasmid	Relevant characteristics[a]	Source/reference
Strains		
E. coli		
XL2-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [*F' proAB lacI*^{q}Z)M15 TN*10* (Tet^R) *amy* Cm^R]	Stratagene
M15	Nal^S Str^S Rif^S Thi$^-$ Lac$^-$ Ara$^+$ Gal$^+$ Mtl$^-$ F$^-$ RecA$^+$ Uvr$^+$ Lon$^+$	Qiagen
S. suis		
10	Virulent serotype 2 strain	Vecht *et al.* (29)
10)FBPS	Isogenic *fbps* mutant of strain 10	This work
Plasmids		
pGEM7Zf(+)	Replication functions pUC, Amp^R	Promega Corp.
pKUN19	Replication functions pUC, Amp^R	Konings *et al.* (14)
pIC19R	Replication functions pUC, Amp^R	Marsh *et al.* (16)
pDL282	Replication functions of pBR322 and pVT736-1, Amp^R, Spc^R	Sreenivasan *et al.* (25)
pIC-spc	pIC19R containing Spc^R gene of pDL282	Lab collection
pQE-30	Replication functions pBR322, Amp^R, expression vector, 6x HIS tag	Qiagen
pQE-30-FBPS	pQE-30 containing the 1.8 kb *fbps* gene	This work
pREP4	Replication functions pACYC, Kan^R, *lac*I gene	Qiagen

pE194	Em^R	Horinouchi and Weisblum (11)
pIVS-E	Replication functions of pWVO1, Spc^R, promoterless *erm* gene of pE194	Smith *et al.* (20)
pIVS-31	pIVS-E containing 200 bp showing homology to *Streptococcus gordonii flpa*	Smith *et al.* (20)
pFBPS7-46	pGEM7Zf(+) containing *Eco*RI-*Eco*RI fragment of *fbps*	This work
pFBPS7-47	pFBPS7-46 in which 382 bp *Sal*I-*Sal*I fragment is replaced by 1.2 kb Spc^R from pIC-spc	This work

[a] Tet^R, tetracyclin resistant; Cm^R, chloramphenicol resistant; Amp^R, ampicillin resistant; Spc^R, spectinomycin resistant; Kan^R, kanamycin resistant; FBPS, fibronectin binding protein *S. suis*.

Table 6. Numbers of re-isolated wild-type (strain 10) and mutant (strain 10)FBPS) bacteria from organs of infected piglets (mean actual inoculation ratio 65% mutant strain).

Organ	Pig number											
	4664			4665			4666			4667		
	w.t.[a]	mut.[b]	perc.[c] mut.	w.t.[a]	mut.[b]	perc.[c] mut.	w.t.[a]	mut.[b]	perc.[c] mut.	w.t.[a]	mut.[b]	perc.[c] mut.
Tonsil	1.77°5	3.29°5	65	4.35°5	2.42°6	85	5.34°4	8.73°4	61	7.94°5	3.96°5	33
pus joint 1	6.75°4	<10	0				1.02°2	5.2°2	84			
pus joint 2	5.15°4	<10	0									
CNS										1.88°5	<10	0

CNS, Central Nervous System

[a] number of wild-type bacteria found (cfu/ml)

[b] number of mutant bacteria found (cfu/ml)

[c] percentage of mutant bacteria calculated as follows: b/(a+b) * 100%

Only relevant organs are depicted.

REFERENCES

Arends, J. P. & Zanen, H. C. (1988). Meningitis caused by *Streptococcus suis* in humans. *Rev Infect Dis* **10**, 131-137.

Axelsson, L. & Holck, A. (1995). The genes involved in production of and immunity to sakacin A, a bacteriocin from *Lactobacillus sake* Lb706. *J Bacteriol* **177**, 2125-2137.

Camacho, L. R., Ensergueix, D., Perez, E., Gicquel B. & Guilhot, C. (1999). Identification of a gene cluster of *Mycobacterium tuberculosis* by signature-tagged transposon mutagenesis. *Mol Microbiol* **34**, 257-267.

Camilli, A. & Mekalanos, J. J. (1995). Use of recombinase gene fusions to identify *Vibrio cholerae* genes induced during infection. *Mol Microbiol* **18**, 671-683.

Chiang, S. L. & Mekalanos, J. J. (1998). Use of signature-tagged transposon mutagenesis to identify *Vibrio cholerae* genes critical for colonization. *Mol Microbiol* **27**, 797-805.

Clifton-Hadley, F. A. (1983). *Streptococcus suis* type 2 infections. *Br Vet J* **139**, 1-5.

Conner, C. P., Heithoff, D. M., Julio, S. M., Sinsheimer R. L. & Mahan, M. J. (1998). Differential patterns of acquired virulence genes distinguish *Salmonella* strains. *Proc Natl Acad Sci USA* **95**, 4641-4645.

Coulter, S. N., Schwan, W. R., Ng, E. Y. W., Langhorne, M. H., Ritchie, H. D., Westbrock-Wadman, S., Hufnagle, W. O., Folger, K. R., Bayer, A. S. & Stover, C. K. (1998). *Staphylococcus aureus* genetic loci impacting growth and survival in multiple infection environments. *Mol Microbiol* **30**, 393-404.

Courtney, H. S., Dale, J. B., Li, Y. & Hasty, D. L. (1994). Cloning, sequencing, and expression of a fibronectin/fibrinogen-binding protein from group A streptococci. *Infect Immun* **62**, 3937-3946.

Darwin, A. J. & Miller, V. L. (1999). Identification of *Yersinia enterocolitica* genes affecting survival in an animal host using signature-tagged transposon mutagenesis. *Mol Microbiol* **32**, 51-62.

Demple, B. (1999). Radical ideas: genetic responses to oxidative stress. *Clin Exp Pharm Phys* **26**, 64-68.

Edelstein, P. H., Edelstein, M. A. C., Higa, F. & Falkow, S. (1999). Discovery of virulence genes of *Legionella pneumophila* by using signature tagged mutagenesis in a guinea pig pneumonia model. *Proc Natl Acad Sci USA* **96**, 8190-8195.

Elliott, S. D. & Tai, J. Y. (1978). The type specific polysaccharide of *Streptococcus suis*. *J Exp Med* **148**, 1699-1704.

Fuller, T.E., Shea, R. J., Thacker, B. J. & Mulks, M. H. (1999). Identification of *in vivo* induced genes in *Actinobacillus pleuropneumoniae*. *Microb Path* **27**, 311-327.

Gottschalk, M., Lacouture, S. & Dubreuil, J. D. (1995). Characterization of *Streptococcus suis* type 2 haemolysin. *Microbiology* **141**, 189-195.

Hensel, M., Shea, J. E., Gleeson, C., Jones, M. D., Dalton, E. & Holden, D. W. (1995). Simultaneous identification of bacterial virulence genes by negative selection. *Science* **269**, 400-403.

Horinouchi, S. & Weisblum, B. (1982). Nucleotide sequence and functional map of pE194, a plasmid that specifies inducible resistance to macrolide, lincosamide and streptogramin type B antibiotics. *J Bacteriol* **150**, 804-814.

Jacobs, A. A., Lo ffen, P. L. W., van den Berg, A. J. G. & Storm, P. K. (1994). Identification, purification and characterization of a thiol-activated hemolysin (suilysin) of *Streptococcus suis*. *Infect Immun* **62**, 1742-1748.

Kolkman, M. A. B., Wakarchuk, W., Nuijten, P. J. M. & van der Zeijst, B. A. M. (1997). Capsular polysaccharide synthesis in *Streptococcus pneumoniae* serotype 14: molecular analysis of the complete *cps* locus and identification of genes encoding glycosyltransferases required for the biosynthesis of the tetrasaccharide subunit. *Mol Microbiol* **26**, 197-208.

Konings, R. N. H., Verhoeven, E. J. M. & Peeters, B. P. H. (1987). pKUN vectors for the separate production of both DNA strands of recombinant plasmids. *Methods Enzymol* **153**, 12-34.

Koskiniemi, S., Sellin, M. & Norgren, M. (1998). Identification of two genes, *cpsX* and *cpsY*, with putative regulatory function on capsule expression in group B streptococci. *FEMS Immun Med Microbiol* **21**, 159-168.

Litwin, C. M. & Calderwood, S. B. (1993). Role of iron regulation of virulence genes. *Clin Microbiol Rev* **6**, 137-149.

Lowe, A. M., Beattie, D. T. & Deresiewics, R. L. (1998). Identification of novel staphylococcal virulence genes by *in vivo* expression technology. *Mol Microbiol* **27**, 967-976.

Mahan, M. J., Slauch, J. M. & Mekalanos, J. J. (1993). Selection of bacterial virulence genes that are specifically induced in host tissues. *Science* **259**, 686-688.

Mahan, M. J., Tobias, T. W., Slauch, J. M., Hanna, P. C., Collier, R. J. & Mekalanos, J. J. (1995). Antibiotic-based selection for bacterial genes that are specifically induced during infection of a host. *Proc Natl Acad Sci USA* **92**, 669-673.

Martinez, J. L., Delgado-Iribarren, A. & Basquero, F. (1990). Mechanisms of iron acquisition and bacterial virulence. *FEMS Microbiol* **75**, 45-56.

Mei, J-M., Nourbakhsh, F., Ford, C. W. & Holden, D. W. (1997). Identification of *Staphylococcus aureus* virulence genes in a murine model of bacteraemia using signature-tagged mutagenesis. *Mol Microbiol* **26**, 399-407.

Miller, J. (1972). *Experiments in Molecular Genetics.* Cold Spring Harbor Laboratory, NY: Cold Spring HarborLaboratory.

Niven, D. F., Ekins, A. & Al-Samaurai, A. A.-W (1999). Effects of iron and manganese availability on growth and production of superoxide dismutase by *Streptococcus suis*. *Can J Microbiol* **45**, 1027-1032.

Payne, S. M. (1993). Iron acquisition in microbial pathogenesis. *Trends Microbiol* **1**, 66-69.

Polissi, A., Pontiggia, A., Feger, G., Altieri, M., Mottl, H., Ferrari, L. & Simon, D. (1998). Large-scale identification of virulence genes from *Streptococcus pneumoniae*. *Infect Immun* **66**, 5620-5629.

Projan, S. J. & Novick, R. P. (1997). The molecular basis of pathogenicity. p. 55-81. In *The Staphylococci in Human Disease,* pp 55-81. Edited by K. B. Crossley & G. L. Archer. Churchill Livingstone, NY.

Quessy, S., Dubreuil, J. D., Jacques, M., Malouin, F. & Higgins, R. (1994). Increase in capsular material thickness following *in vivo* growth of virulent *Streptococcus suis* serotype 2 strains. *FEMS Microbiol Lett* **115**, 19-26.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual,* 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Segers, R. P. A. M., Kenter, T., de Haan, L. A. M. & Jacobs, A. A. C. (1998). Characterization of the gene encoding suilysin from *Streptococcus suis* and expression in field strains. *FEMS Microbiol Lett* **167**, 255-261.

Serhir, B., Dugourd, D., Jacques, M., Higgins, R. & Harel, J. (1997). Cloning and characterization of a dextranase gene (*dexS*) from *Streptococcus suis*. *Gene* **19**, 257-261.

Smith, H. E., Vecht, U., Gielkens, A. L. J. & Smits, M. A. (1992). Cloning and nucleotide sequence of the gene encoding the 136-kilodalton surface protein (muramidase-released protein) of *Streptococcus suis* type 2. *Infect Immun* **60**, 2361-2367.

Smith, H. E., Reek, F. H., Vecht, U., Gielkens, A. L. J. & Smits, M. A. (1993). Repeats in an extracellular protein of weakly pathogenic strains of *Streptococcus suis* type 2 are absent in pathogenic strains. *Infect Immun* **61**, 3318-3326.

Smith, H. E., Wisselink, H. J., Vecht, U., Gielkens, A. L. J. & Smits, M. A. (1995). High-efficiency transformation and gene inactivation in *Streptococcus suis* type 2. *Microbiol* **141**, 181-188.

Smith, H. E., Vecht, U., Wisselink, H. J., Stockhofe-Zurwieden, N., Biermann, Y. & Smits, M. A. (1996). Mutants of *Streptococcus suis* types 1 and 2 impaired in expression of muramidase-released protein and extracellular protein induce disease in newborn germfree pigs. *Infect Immun* **64**, 4409-4412.

Smith, H. E., Damman, M., van der Velde, J., Wagenaar, F., Wisselink, H. J., Stockhofe-Zurwieden, N. & Smits, M. A. (1999). Identification and characterization of the *cps* locus of *Streptococcus suis* serotype 2: the capsule protects against phagocytosis and is an important virulence factor. *Infect Immun* **67**, 1750-1756.

Stockhofe-Zurwieden, N., Vecht, U., Wisselink, H. J., van Lieshout, H. & Smith, H. E. (1996). Comparative studies on the pathogenicity of different

Streptococcus suis serotype 1 strains. In *Proceedings of the 14th International Pig Veterinary Society Congress*, pp 299. Edited by P. G. Monetti & G. Vignola. Bologna, Italy.

Van der Vossen, J. M. B. M., van der Lelie, D. & Venema, G. (1987). Isolation and characterization of *Lactococcus lactis* subsp. *cremoris* Wg2-specific promoters. *Appl Environ Microbiol* **52**, 2452-2457.

Vecht, U., van Leengoed, L. A. M. G. & Verheyden, E. R. M. (1985). *Streptococcus suis* infections in pigs in The Netherlands (part one). *Vet Quart* 7: 315-321.

Vecht, U., Arends, J. P., van der Molen, E. J. & van Leengoed, L. A. M. G. (1989). Difference in virulence between two strains of *Streptococcus suis* type 2 after experimentally induced infection of newborn germfree pigs. *Am J Vet Res* **50**, 1037-1043.

Vecht, U., Wisselink, H. J., Jellema, M. L. & Smith, H. E. (1991). Identification of two proteins associated with virulence of *Streptococcus suis* type 2. *Infect Immun* **59**, 3156-3162.

Vecht, U., Wisselink, H. J., van Dijk, J. E. & Smith, H. E. (1992). Virulence of *Streptococcus suis* type 2 strains in newborn germfree pigs depends on phenotype. *Infect Immun* **60**, 550-556.

Vecht, U., Stockhofe-Zurwieden, N., Tetenburg, B. J., Wisselink, H. J. &. Smith, H. E. (1997). Virulence of *Streptococcus suis* type 2 for mice and pigs appeared host-specific. *Vet Microbiol* **58**, 53-60.

Wang, J., Mushegian, A., Lory, S. & Jin, S. (1996). Large-scale isolation of candidate virulence genes of *Pseudomonas aeruginosa* by *in vivo* selection. *Proc Natl Acad Sci USA* **93**, 10434-10439.

Yamashita, Y., Shibata, Y., Nakano, Y., Tsuda, H., Kido, N., Ohta, M. & Koga, T. (1999). A novel gene required for rhamnose-glucose polysaccharide synthesis in *Streptococcus mutans*. *J. Bacteriol* **181**, 6556-6559.

Young, G. M. & Miller, V. L. (1997). Identification of novel chromosomal loci affecting *Yersinia enterocolitica* pathogenesis. *Mol Microbiol* **25**, 319-328.

Zhao, H., Li, X., Johnson, D. E. & Mobley, H. L. T. (1999). Identification of protease and *rpoN*-associated genes of uropathogeneic *Proteus mirabilis* by negative selection in a mouse model of ascending urinary tract infection. *Microbiol* **145**, 185-195.

Further references

1. **Allgaier A., R. G., H. J. Wisselink, H. E. Smith, and P. Valentin-Weigand.** 2001. Relatedness of *Streptococcus suis* isolates of various serotypes and clinical backgrounds as evaluated by macrorestriction analysis and expression of potential virulence traits. J. Clin. Microbiol. **39**:445-453.
2. **Allen, A. G., S. Bolitho, H. Lindsay, S. Khan, C. Bryant, P. Norton, P. Ward, J. Leigh, J. Morgan, H. Riches, S. Eastty, D. Maskell.** 2001. Generation and characterization of a defined mutant of *Streptococcus suis* lacking suilysin. Infect. Immun. **69**:2732-2735.
3. **Arends, J. P., and H. C. Zanen.** 1988. Meningitis caused by *Streptococcus suis* in humans. Rev. Infect. Dis. **10**:131-137.
4. **Charland, N., V. Nizet, C. Rubens, K. Kim, S. Lacouture, and M. Gottschalk.** 2000. *Streptococcus suis* interactions with human brain microvascular endothelial cells. Infect. Immun. **68**:637-643.

5. **Chia, J. S., C. Y. Yeh, and J. Y. Chen.** 2000. Identification of a fibronectin binding protein from *Streptococcus mutans*. Infect. Immun. **68**:1864-1870.

6. **Clifton-Hadley, F. A.** 1983. *Streptococcus suis* type 2 infections. Br. Vet. J. **139**:1-5.

7. **Courtney, H. S., J. B. Dale, an D. L. Hasty.** 1996. Differential effects of the streptococcal fibronectin-binding protein, FBP54, on adhesion of group A streptococci to human buccal cells and Hep-2 tissue culture cells. Infect. Immun. **64**:2415-2419.

8. **Courtney, H. S., Y. Li, J. B. Dale, and D. L. Hasty.** 1994. Cloning, sequencing, and expression of a fibronectin/fibrinogen-binding protein from group A streptococci. Infect. Immun. **62**:3937-3946.

9. **Fuller, T. E., S. Martin, J. F. Teel, G. R. Alaniz, M. J. Kennedy, and D. E. Lowery.** 2000. Identification of *Actinobacillus pleuropneumoniae* virulence genes using signature-tagged mutagenesis in a swine infection model. Microb. Pathog. **29**:39-51.

10. **Greene, C., D. McDevitt, P. François, P. Vaudaux, and T. J. Foster.** 1995. Adhesion properties of mutants of *Staphylococcus aureus* defective in fibronectin-binding proteins and studies on the expression of *fnb* genes. Mol. Microbiol. **17**:1065-1076.

11. **Horinouchi, S., and B. Weisblum.** 1982. Nucleotide sequence and functional map of pE194, a plasmid that specifies inducible resistance to macrolide, lincosamide and streptogramin type B antibiotics. J. Bacteriol. **150**:804-815.

12. **Joh, D., E. R. W. Wann, B. Kreikemeyer, P. Speziale, and M. Höök.** 1999. Role of fibronectin-binding MSCRAMMS in bacterial adherence and entry into mammalian cells. Matrix Biol. **18**:211-223.

13. **Kawabata, S., E. Kunitomo, Y. Terao, I. Nagakawa, K. Kiruchi, K.-I. Totsuka, and S. Hamada.** 2001. Systemic and mucosal immunizations with fibronectin-binding protein FBP54 induce protective immune responses against *Streptococcus pyogenes* challenge in mice. Infect. Immun. **69**:924-930.

14. **Konings, R. N. H., E. J. M. Verhoeven, and B. P. H. Peeters.** 1987. pKUN vectors for the separate production of both DNA strands of recombinant plasmids. Methods Enzymol. **153**:12-34.

15. **Mahan, M. J., J. M. Slauch, and J. J. Mekalanos.** 1993. Selection of bacterial virulence genes that are specifically induced in host tissues. Science **259**:686-688.

16. **Marsh, J. L., M. Erfle, and E. J. Wykes.** 1984. The pIC plasmid and phage vectors with versatile cloning sites for recombinant selection by insertional inactivation. Gene **32**:481-485.

17. **Miller, J.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory. Cold Spring Harbor, N.Y.

18. **Peacock, S. J., T. J. Foster, B. J. Cameron, and A. R. Berendt.** 1999. Bacterial fibronectin-binding proteins and endothelial cell surface fibronectin mediate adherence of *Staphylococcus aureus* to resting human endothelial cells. Microbiology **145**:3477-3486.

19. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor. N.Y.

20. **Smith, H. E., H. Buijs, R. de Vries, H. J. Wisselink, N. Stockhofe-Zurwieden, and M. A. Smits.** 2001. Environmentally regulated genes of *Streptococcus suis*: identification by the use of iron-restricted conditions *in vitro* and by experimental infection of piglets. Microbiol. **147**:271-280.

21. **Smith, H. E., H. Buijs, H. J. Wisselink, N. Stockhofe-Zurwieden, and M. A. Smits.** 2001. Selection of virulence-associated determinants of *Streptococcus suis* serotype 2 by *in vivo* complementation. Infect. Immun. **69**:1961-1966.

22. **Smith, H. E., M. Damman, J. van der Velde, F. Wagenaar, H. J. Wisselink, N. Stockhofe-Zurwieden, and M. A. Smits.** 1999. Identification and characterization of the *cps* locus of *Streptococcus suis* serotype 2: the capsule protects against phagocytosis and is an important virulence factor. Infect. Immun. **60**:1750-1756.

23. **Smith, H. E., U. Vecht, H. J. Wisselink, N. Stockhofe-Zurwieden, Y. Biermann, and M. A. Smits.** 1996. Mutants of *Streptococcus suis* types 1 and 2 impaired in expression of murimidase-released protein and extracellular protein induce disease in newborn germfree pigs. Infect. Immun. **64**:4409-4412.

24. **Smith, H. E., H. J. Wisselink, U. Vecht, A. L. J. Gielkens, and M. A. Smits.** 1995. High-efficiency transformation and gene inactivation in *Streptococcus suis* type 2. Microbiology **141**:181-188.

25. **Sreenivasan, P. K., D. L. LeBlanc, L. N. Lee, and P. Fives-Taylor.** 1991. Transformation of *Actinobacillus actinomycetemcomitans* by electroporation, utilizing constructed shuttle plasmids. Infect. Immun. **59**:4621-4627.

26. **Staats, J. J., I. Feder, O. Okwumabua, and M. M. Cheganppa.** 1995. *Streptococcus suis*: past and present. Vet. Res. Comm. **21**:381-407.

27. **Vecht, U., J. P. Arends, E. J. van der Molen, and L. A. M. G. van Leengoed.** 1989. Differences in virulence between two strains of *Streptococcus suis* type 2 after experimentally induced infection of newborn germfree pigs. Am. J. Vet. Res. **50**:1037-1043.

28. **Vecht, U., L. A. M. G. van Leengoed, and E. R. M. Verheyen.** 1985. *Streptococcus suis* infections in pigs in The Netherlands (part I). Vet. Q. **7**:315-321.

29. **Vecht, U., H. J. Wisselink, J. E. van Dijk, and H. E. Smith.** 1992. Virulence of *Streptococcus suis* type 2 strains in newborn germfree pigs depend on phenotype. Infect. Immun. **60**:550-556.

30. **Vecht, U., H. J. Wisselink, M. L. Jellema, and H. E. Smith.** 1991. Identification of two proteins associated with virulence of *Streptococcus suis* type 2. Infect. Immun. **59**:3156-3162.

Claims

1. A method for modulating virulence of a *Streptococcus* comprising modifying a genomic fragment of said *Streptococcus* wherein said genomic fragment comprises at least a functional part identifiable by hybridization in *Streptococcus suis* to a nucleic acid or fragment thereof as shown in Fig 6 and obtaining a clone wherein said genomic fragment has been modified.
2. A method according to claim 2 wherein said genomic fragment comprises at least a functional part of a gene expression of which in *S. suis* can be environmentally regulated by iron-restricted conditions.
3. A method according to claim 1 and/or 2 wherein said genomic fragment comprises at least a functional part of a gene which is expressed in a with a wild-type *S. suis* infected pig.
4. A method according to claim 3 wherein said gene encodes a fibronectin/fibrinogen binding protein.
5. A method according to anyone of claims 1 to 4 for modulating virulence of a *Streptococcus suis*.
6. A method according to anyone of claims 1 to 5 wherein said method comprises functionally deleting the expression of at least said functional part of said gene by said *Streptococcus*.
7. A clone of a Streptococcus obtained by a method according to anyone of claims 1 to 6.
8. A method for assaying virulence of a *Streptococcus* comprising assaying a genomic fragment of said *Streptococcus* wherein said genomic fragment comprises at least a functional part of a fragment identifiable by hybridisation in *Streptococcus suis* to a nucleic acid or fragment thereof as shown in Fig .6.
9. An isolated and/or recombinant nucleic acid derivable from *Streptococcus* and identifiable by hybridisation in *Streptococcus suis* to a nucleic acid or fragment thereof as shown in Figure 6.
10. A vector comprising a nucleic acid according to claim 9
11. A host cell comprising a nucleic acid according to claim 9 or a vector according to claim 10.
12. A host cell according to claim 11 comprising a *Streptococcus*.

13. A vaccine comprising a clone according to claim 7 or a nucleic acid according to claim 9 or a vector according to claim 10 or a host cell according to claim 11 or 12.

14. A protein or fragment thereof encoded by a nucleic acid according to claim 9.

15. An antibody directed against a protein or fragment thereof according to claim 14.

16. An antigen comprising a protein or fragment thereof according to claim 14

17. A diagnostic test comprising an antibody according to claim 15.

18. A vaccine or diagnostic test comprising an antigen according to claim 14.

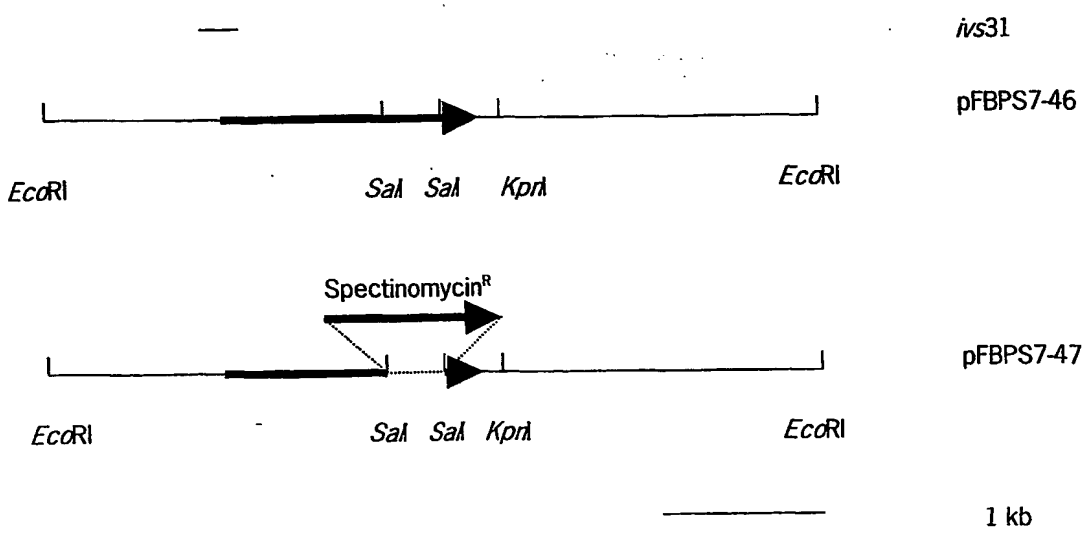
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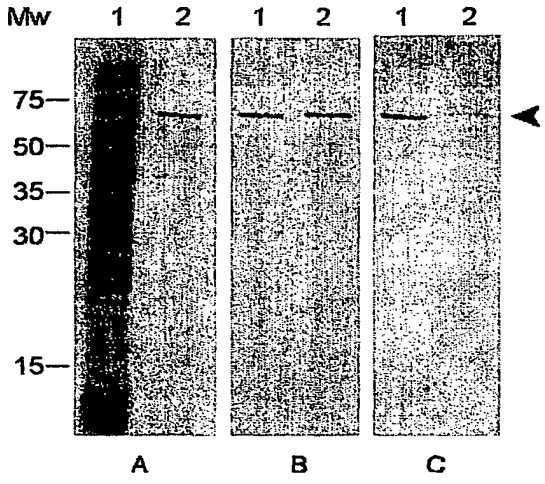
Fig 1



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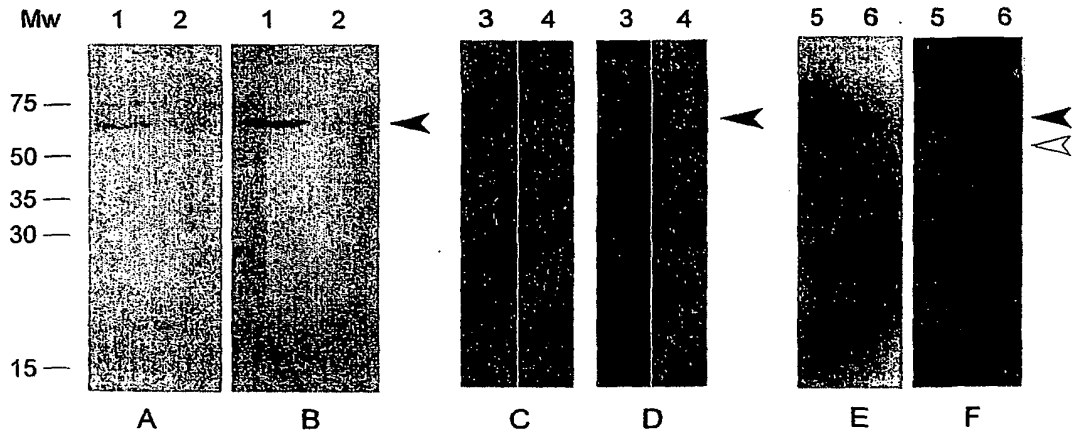

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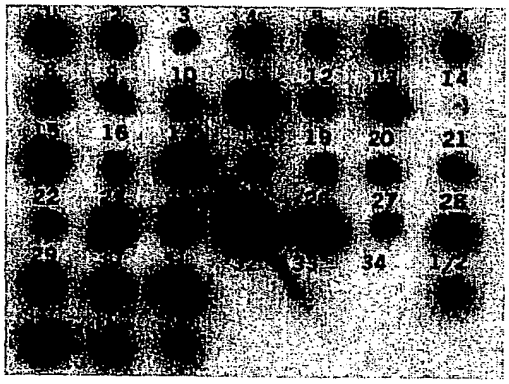

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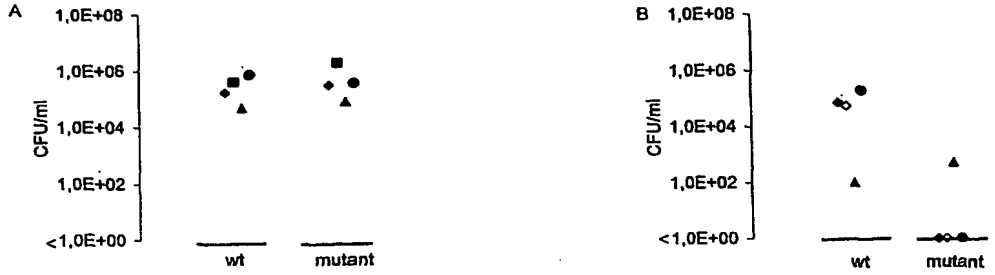
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<1,0E+00
CFU/ml
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CFU/ml
wt
mutant

Fig 5.

Figure 6 A

iri 1, 6, 22

GAAACGTCAGTAAGGTATAAATTCCTAGAAGTTNTTGGTAAACCAAATCAATNATTGGAATCAATTGGGGAAGCAGGGAA
TCATCAATTTTTCTTTTTAGATATTGAAATAAAAGGAGAAGAAAAGAAAGGAATGGAAATCGCTAAAGAAATCCGGGCTC
GAGATCCTTATGCTGCTATTGTCTTTGTAACAACTCACTCAGAATTNATGCCAGTAACATATCGTTATCAGGTTTCTGCT
TTAGATTTTATAGATAAAGGCCTGGAGGATCGTGACTTTCAAAAGGCAGTATCAGANGTCTTAGTGCATGCTTTTGAAAA
TATCGATCATACTNT

Figure 6B

iri 10, 20

GNTNGGCGGTTTTCCAGCCCGTTNATGCAGTTNGGTTGTTGCTNNGAACAGCAAGAATATCCCCCNGAACAACATAATCA
GGTCGNGTCCGGAGAAGGAGAAATCCCATGACGGTAAATNGCGGTTTGGTCAGNGTGACCATCCATGAAATCAGCGACAG
CAGCCCCAGACGCATAATCAGCGCCAGTGATAACCCCAGCAAACGCGCTTTATCGNGTTGTTTTGGCGGCAGTTTGTCAG
CAAGAATGGCGATGAAGACCAGGTTATAGATACCCAGCACAATTTCGAGAACAACCAGCGTGAGTAGCCCCGCCCAAATT
GAGGGGTCCATTAAGNCNTACGAAANT

Figure 6 C

iri 11

TTCATCCTNGTCGAGNGGGGAAATGGGGCACAGTTGTTTTCCAATTGATAGAATTTTTAAGAACCTATATATANAAACAT
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TTGGAGGTTTGTCAGATGAATGTGCAAGAAAATGTCTTACCGAGTATAGAATTATTGGTTTTGAAACGTGATGGACGGAC
AGTATCCTTTGACCANGATAAGATTTTTTNTGCTCTTCAGCGGGCAAACCAAGAATTAGAACATCCTGTTTCAGNGGCAG
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Figure 6 D

iri 13, 15, 27

GGAGGNGGTTANACNGGCATTTGCAGATGCCGAATTTATCTAATGATTTTGTAGAAGAGTGGTNGCATNGAACAACCCTC
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ATAGACTAGTTAATATTTGCAGAAACACTTGNAACACAATTAAAGAAACTGGTANTATTGAATAGTAAGCGTAAAAACTT
TACTACACTTCAGTCACTATTTTACNTCAA

Figure 6 E

iri 14

TATTNNTAGTGTACTATAGAAAAAACTAACNTACCACAANACGTGATAGGTTAGTTAANTTAATGACATTGGGCTNTTTG
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Figure 6 F

iri 16

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GTGTAACAAGGANATCAAGTTCAAGGCTTTNTTGGANTACGNTATGAACTTGGGTGCGGACTATGTGGCGACAGGGCACT
ACGCTCAGGTNACCCGCGACGANGACGGCACCGTTCATATGCTGCGTGGGGCAGATAATGGTAAGGACCAGACCTATTTC
CNCAGCCAACTCTCACGNNAAC

Figure 6 G

iri 18

CTGTCTAACCACCCTAACATNGCATANTCCTCCTTTTTTATCTATTTTATCAAAAAATCGGNGCTTTTCTACCATTTGTC
AAGTTCATCAAGGTATTTGACGAAAAATANTNNGTGTCTCGTCATCCAAATAAGGAAATTGTTTTATTTTGGACTAAAGT
TACGTGTAAAAAGNGCATACAAAACCAACACCTTNTGTTGNAATTTTTTGATAAGGTGTTACAATGATAGAGCATAAACA
GTTTTACCGATTTTGGGTNGAAGCGTAATCGTNAAATTTGTTATGCNTAATGAGGTAATACATTGTCCGAATGAGACGAT
GTATGGAGGCGATCGNANGN

Figure 6 H

iri 2

ANCCTTTCNNGGCCCNATGGATGTTTCGNGGAGAAAATTGGAAAAATAATCGGGATTATCCAGGGAAATNTCAAAATCAC
AGTTATCCGTGAAACAAGAGCAACNGANGTTGCNAAGTAAATGTAGAAGAGTCCGAAGGGCTCTTTTTNTACTGGCTCAA
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TTACACTAGAATGAAAGATTTAAAAGGAGATATCATGAAAGAGCGAGGCTTACTCATNGTCTTTTCTGGTCCATCTGGTG
CNGGAAAAGGAACAGTTCGAAAGGNAATTTNTGAA

Figure 6 I

iri 23

GNTCTNGTAGTAGATAAGATTGAAACGCCAAATTCNTCGTAACAAAACCAAGATTGAACGAAAGCNTCGTCAAAAAGTGG
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TGACGGTACAACAAATGTATTGTATAGACGCGAAGATGGAGATTTGGGTCTTCTAGAGNTACGTCAATAAAGATAATAAA
ACAGCNCNANCGNANNN

Figure 6 J

iri 24

AGAAGAGAAATGGGGGAACCTGGNAGTTCTACAAGAAATTAGTTTTGAAGAACAGGACGGGGCTAGTCTATTTGCGAAAA
CGCAAGAGTTCAGCAAATTCTTGCTTTTTTTGATATAATGGTAGAAGCAGTTTTAAGAGGTATCCAGGTATGAATATTCA
ACAATTACGCTACGTTGTAGCCATTGCAAACAGTGGTACATTTCGAGAGGCGGCTGAGAAAATGTANGTGTCCCAGCCTA
GNTNGTCCATTTCCATTCGTGATTTGGAAAAAGAGTTAGGTTTTCAAATTTTTNGCCGAACTAGTTCAGGAACTTTTTTG
ACACAAAAAGGGATGGNACTCACGGAGATA

Figure 6 K

iri 29

GCGTATGGGGGAAATTGCCCAAGATGTTANCCGGTGACAAAATCACAGATGCAGCCCGTAATCANGCAAAAGAATTAGTA
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GGCGAANTCNTAG

Figure 6 L

iri 3

AGGNTGCTANGAAAAAATTGGCTCACAAATCATTTCTTTTANTTGACGATTGCCTTTCTTTNGATTTNGGTGATTTACTT
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CNAAATCTNACAGAAAGGAGN

Figure 6 M

iri 32

GAATCGAATTGGAGNTCGCCCCTCAAACGGCTGGCATATCTTTTCAATCCTTATCTNTNAGTCGCAAGCGACAAGGANTA
GGGATNATATAATCTCCTGAGAATACTGGACTCACTGAGTCTGGTATTTTCATTTTATGCTATAATGGTTTCATGACAAA
TCGAATTTTAGATATGGAACAAATGCAGGACGAGGAATATGTCGNGCGTACCCTGCGTCCNCAGAAATTAAACGAATACA
TCGGTCAGGACAAGGTTAAGGACC

Figure 6 N

iri 34

ACAGTAGCCTATGAANTCTTGGAAGNAAGCGGGAAGAAGCAAACCATTAGTTTCGACCAAATTTTAGTTCCCATAGGAGG
AGGTGGTCTGGTTGCAGGCGTTTCGGCCTATNTGAAAGAACATGCACCTGAAATTANGATTGTTGGTGTTGAAGCAAGTG
GGGCACGGTCAATGAAAGCGGCTTTNGATAAAGGTCGTCCGGTTNAATTAGACCAAATTGATAAATTTGCTGACGGTATT
GCGGTACAGAAAGTCGGTAAGTCGACCTACGAAGTGGCTCGGAAATACGTAGATCGCCTGATTNGTNTGGATGAAGGGTG
GATTTCCGGGANTATTTT

Figure 6 O

iri 4

AAAATGGCAGGGGGGACCCAAGGGAANTCTTTTCTGATATCAAGGGACAACCTGGTCAGTCAGNTGGNTCAANTACAAGC
CTTACCACTNGAACAAATANTCGAAAACCGTTATCAACGCTTTAGAAAATANTAGGAAGACCTAGNATTTTTTTGATAGA
TTTGATACAATGGATAAAATAATTTCAGGAGGTTTTCCATGTTAGTAAAAGCAGATCTATCAAACGCAGNAGAATTGCTA
CNTATTCAGCNCCGAGCATTTGCGGCTTTATATAAAACCTATCAGGACCAGTACAACCCTGCCATTGNAACTATGGACTA
TTTCCAATCACGCTTTGCACGACCAAATTGT

Figure 6 P

iri 7

GAAATTGATGGGCATCTTTGGTATTAATAGGAACTCCATGGCTCAATCTTCTTCGGTTTATTGGTAATAGTAGTTACCGT
CANGAANAAATCGCAAAGTATAAAAAAGTGCTGTGAAGAGAAAAAAAAGAAAATAAGAATCTTTCTAAACAAGATAAGAGCC
GTCAGGCTCTTTTTTNGATATAATATAGTGGATATGGTTAATTAAAATTGTCAGAAAAGACTATTTTANAGATTAACACT
CTCTGAAAATCNTCATTAACAAGAAAAGAGGCNGGGCTCAAGCCCCGCATCACNTCTCAAAGTTAGCGTCAACATCTCAG
CGCAGTAGTGGTNGATTGGGTTTAACAGTCCAGTGGAGTGTC

Figure 6 Q

iri 8, 26

TTCGGAATCCTTCTNTCTCCATTGGAACAGGGATACAAAGGGACGTTAAGGAAATCCGTANGAAAATAGGAAATTGACGC
AGTGTGCTANACACACAGGGAAGTTTATCTTTTTCCACTAGGATTTTAGTCCGTGTTCAACTAAGATACGAGATATGTTC
TGGTTTACCAGAAATTTCNGNAGAAAATTAGGAGACTGACGCTGAGTGTTAACACTCAAGGAAGGCTATCTATTTTTCTA
AGAAATTAATCTCGAGTTCAATTTCTTNTGATTAGTAAATAAATGAATTGTATCTATTTTTTGGGGTATCGCCAAGCGGT
AAGGCAAGGGACTTTGACTCCCTCATGCGCCGGTTNGCATCC

Figure 6 R

ivs 1

AATGATGTTTGATAAACACGCCAATCTCAAATACAAATTTGGTAATCGTCATTTCTGGGCAGAGGGATATTATGTAAGTA
CGGTTGGACTAAATGAAGCCACAATTAAGAAATATATACAAGAACAGGAAAAACATGATATAGCACTTGATAAGTTGAGT
GTAAAAGAGTATGAAGATCCCTTTAGGGATAATGGCAAGTAGTACGAATGCCTCTTTAAGAGGCTAGTGACGAGTCAAAA
GCAGTGAGGCTTGAACAAAGTGAAAGCCAGCGTCTTTAGGCGCTGGCTGGTGATGTGGGCTTATAGCCCTTGTTCAAACC
ACCCGTTTGACGGGTGGTCATGATTTTTTTTGAATATTTTTCACTATTTTGTTTTACAAACTAGCCACCTTGTGTTAGAC
TATAG

Figure 6 S

ivs 11

TACCACCATATCACCAATATCACGCGCCCAGATGCGCCAATCGAAGTGGTGGATGTGGCTGGTTCCCTTTGTGAAAACAA
CGACAAGTTTGCGGTCAATCGTGAATTACCACGGGTAGAAGTAGGAGACACCTTGGTCATTCATGACAGTGGGGCCCACG
GCTTCTCCATGGGCTACAACTACAACGGTCGTCTGCGTTCTTCTGAAATCCTTTTGCAGGAAGATGGCACAGCGCGGATG
ATTCGTCGTGCTGAAACACCAGAAGACTATTTCGCAACTATTTACGGTTTTGATTTTGACAGGTAAGTCTTGGAAAAGAC
TAGGGAATTTGGTATAATAGGGTTATTGAAAGATTGTTAAAAACAATCAGAAGTATACTTTTTAGAAGAGTCAGGAGATT
GACAGATGAA

Figure 6 T

ivs 15

GGGCTATGGTATAATTAAAAGACATGTATAGTCAGAATGAAAATGAATTGATTGCCATTGGTGAGAGAATTGGAAAGGCC
TGTAAGCCAAATCAAGTTCTAGTATTATCAGGGGATTTGGGTGCTGGGAAAACAACTCTGACCAAGGGTTTGGCCAAGGG
GTTAAAAATTGAACAGATGATTAAGAGTCCTACTTATACGATTGTTCGAGAGTATGAGGGGGCCATGCCGCTCTATCACT
TAGATGTTTATCGAATTGGAGATGACCCTGACTCGATTGATTTGGATGATTTTCTCTATGGAGGAGGTCTAACGGTTATC
GAGTGGGGAGAATTACTGGATGTCAGTCTATTTGATGACTATTTGCTCATTCGTATAGAGAAAGAGGGAGATGGTCGACG
ATTGACAGTCGA

Figure 6 U

ivs 16

GAAAATTGTTGTTGTTTTGGAACACTAGTAGACCAGAGGCTTCTAGTAAGGTAGTTGTGCTCACTGAGGAGGGGGAAGGA
TGATGGAAGTTGAGAAAAGGAGTAAGGATTATGCTCGTATGTTTGACCAGCAAGTCGGTCTTTATGAAGACTATGCTCGT
GGACATGGACTCAATGCAAAATGTTTATCCATTCTCATGTGGATTTATTATAATCCCGGAGGTGTGACGCAAAACTGGGT
CAGTAAGAAGACCTATTCAAGCAAACAAGTTGTCAATGCTACTGTAAAGAAATTTTTGGATGGAGGCCTGGTAGTTCTAG
AGGAGAATCCAGCAGATAAGCGACATAAGAAAATTAAATTGACAGAGGAGGGGCAACAATTTGCTAGTCGCATTTTGGAT
CCCTTAGAGGAGGCGGAAAATAAGGCGCTGTCTCAACTCAGTCAGGAGG

Figure 6 V

ivs 18

GCGTTTTGGAACAAGTACGTTAAGAGAAACCTAGAGAAATCTAGGGTTTTTGCTTTTATATATCTTTACATTGTTTAAAG
AAAATAGCATTTCAAAAACTTTTTGAAAAAAATGTGATATTCTGAGCATATTTTTTGAAATCGGTAACATTTATATTGTA
TAATATAGTTCGTAAAAAAATATATTTTCGAAAGTGAGATTTTACATTATGGCTAAAATCGTTGTTGTCGGTGCTAACCA
TGCTGGTACTGCCGCAATCAAAACTATGTTGACAAATTATGGTCAAGAAAATGAAATCGTTGTATTTGACCAAAACTCAC
ATATTTCATTCTTGGGTTGTGGTATGGCTTTGTGGATCGGTGAGCAAATTGGCGGTCCTGAAGGACTCTTCTACTCAAAC
AAAGAAGAGT

Figure 6 W

ivs 19

TCGTTCCATTTGCTGGTGAAATGCCCAGCAATACGCTTCNTAGCAATAGAAGAACCAAATAGATGGCACTCAATTTCATG
AGGAAGAACAGAAGAGTAAAAAGCCTGTCTAACCACCCTAACATAGNATATTCCTCCTTTTTCATCTATTTTATCAAAAA
ATCGGTGCTTTTCTACCATTTGTCAAGTTCATCAAGGTATTTGACGAAAAATATTTTGTGTCTCGTCATCCAAATAAGGA
AATTGTTTTATTTTGGACTAAAGTTACGTGTAAAAAGTGCATACAAAACCAACACCTTATGTTGAAATTTTTTGATAAGG
TGTTACAATGATAGAGCATAAACAGTTTTACCGATTTTGGGTTGAAGCGTAATCGTAAAATTTGTTATGCATAATGAGGT
AATACATTGTCCGAATGAGACGATGTATGGAGGCAAT

Figure 6 X

ivs 2, 4, 28

AAGACGGCGTCAAGGATGACAATCTTGTGGTGACGACCACCCAGAAACTGGCGTAGCNTTTACCGTGGCCGGAATCATGA
TCGCGGTTTTCCAGCCGTTCATGCAGTTCGGTTGTTGCTTTGAACAGCAAGAATATCCCCCCGAACAACATAATCAGGTC
GCGTCCGGAGAAGGAGAAATCCATGACGGTAAAATAGCGGTTTGGTCAGCGTGACCATCCATGAAATCAGCGACAGCAGC
CCCAGACGCATAATCAGCGCCAGTGATAACCCCAGCAAACGCGCTTTATCGCGTTGTTTTGGCGGCAGTTTGTCAGCAAG
AATGGCGATGAAGACCAGGTTATCGATACCCAGCACAATTTCGAGAACAACAAGCGTGAGTAGCCCCGCCCAAATTGAGG
GGTCCATTAAGAATTCC

Figure 6 Y

ivs 20

AGTTCAGATGTTCGGTTTAGGAATTGCCGGCGTCTGGCTGTCGATTTTGATGGACCTGCTCTTGCGAGCGATTTTTCTGA
CTTGGAGGTTTATTGTGCAAACACGAAAACTGGCTGAATAGGCTAGTTTTTTGGTATAATATCAGTAGAATGATAAAAAG
GAGATAATCAGATGAAAACCATTCACACAGATAAGGCACCTGCAGCAATTGGCCCATACGTTCAAGGGAAGGTTGTTGGA
AATTTCCTATTTGCCTCTGGTCAAGTTCCTTTGTCACCTGAAACTGGTGAAGTGGTTGGTGAAACCATTCAGGAGCAGAC
TGAGCAAGTCTTGAAAAATATCGCAGCAATTTTATCAGAAGCAGGAACAGACTTTGACCATGTGGTGAAGACGACTTGTT
TCCTAAAAGATATGAATGATTTTGTAGCCTTTAATGAAGTTTAT

Figure 6 Z

ivs 23, 24

TCTGCACTGTTGCGCTGCCTATAAGTTCTACGTTCAGTAGTAGATGAAATGTTCAGAGGAAGTGGTATGGGTTCCAACTT
AGTAAAATTAGTCATTGATGATTTGGCGAACAGAAATTCCAAAGCCTTTCAAATCGCAGTTGAAGAAGAGAAATTGGGAA
CCTGGAAGTTCTACAAGAAATTAGTTTTGAAGAACAGGACGGGCTAGTCTATTTGCGAAAACGCAAGAGTTCAGCAAATT
CTTGCTTTTTTTGATATAATGGTAGAAGCAGTTTTAAGAGGTATCAGGTATGAATATTCAACAATTACGCTACGTTGTAG
CCATTGCAAACAGTGGTACATTTCGAGAGGCGGCTGAGAAAATGTATGTGTCCCAGCCTAGTTTGTCCATTTCCATTCGT
GATTTGGAAAAAGAGTTAGGTTTTCAAATTTTTAGCCGAACTAGTTCAGGAACTTTTTTGACACAAAAAGGGATGGAATT

Figure 6 A1

ivs 25

GGAGATAGCAATGCTTAATATTTTTGTATTAGAAGATGATTTTTTTCAGCAGAGCAGGTTAGAAAATGCTATTAGGCAGT
GTGTTGAAGAAACGTCAGTAAGGTATAAATTCCTAGAAGTTTTTGGTAAACCAAATCAATTATTGGAATCAATTGAGGAA
GCAGGGAATCATCAATTTTTCTTTTTAGATATTGAAATAAAAGGAGAAGAAAAGAAAGGAATGGAAATCGCTAAAGAAAT
CCGGGCTCGAGATCCTTATGCTGCTATTGTCTTTGTAACAACTCACTCAGAATTTATGCCAGTAACATATCGTTATCAGG
TTTCTGCTTTAGATTTTATAGATAAAGGCCTGGAGGATNGTGACTTTCAAAAGGCAGTATCAGATGTCTTAGTGCATGCT
TTTGAAAATATTGATCAT

Figure 6 B1

ivs 29

GGCAAGGGTGGGTAAATTTCTAATTGGTGACAAGGCACTTGAATTCTACCCAGATAGCAACGTTGAACGCTATATCCAGA
TTCCTTGGTCAGAAATGACTAGCATTGGCGCAAAACGTTTCTGGCAAAGCAATCAGCCGTCATTTTGAAATTTATACAGA
GAAAAGTCGATTTCTTGTTGGCATCTAAAGATTCTGGTAAGATTCTTAAAATTGCCCGTGAGCATATCGGCAATGAAAAA
GTTGTGAAATTACCGACTCTTATGCAAACAATCGGCAGAAAAATTTCGAATCTATTTGCCAAAAAATAAAAATTCAAGTA
TAATAGTAGAAACGGATAAGTAGCATCTGGCTCCTTCCAGAAAGTCTGCGGTCGCTGTGAGCAGATAGGAAAAAGTTGTG
AAATTCTACCGTTATGAAATTATCAAAATACAATCAAGTGCACAGA

Figure 6 C1

ivs 3

GGATTATCTACTATAAGCAGTATTCAGAAGGGCATGAGGACAAGAAATCCTACAAGATTCTACAAGAAGTAGGCATGAGC
CAGAAGGCTGTCAAGAAAACAATTAACTCCCAAACACTTACGGTCTTCTTTATGCCTTTGGTCATGGCGACCCTACACTT
TGTCATCGCCCTTATCATGCTCAAGCAAATGCTACTAAGTTTTGGTGTTACCTCATCACTAATGATTTACACAGTCAGTG
GCATCACCCTACTGGCAGTCACTCTGATTTACTTTGTCATTTACAAGTGGACTAGTCGCACTTATTATCGCATTATTGAA
CGGTAGCAGAAGTCTCGCCTTGTGCGAGATTTCTTGCTTTTTCAGGGAAATGGTGTTACAATGGTAATACCAAAGGAATA
CTCGAAGAGGTGAGAA

Figure 6 D1

ivs 31

ACGAAAATCGATGGATCCATGCATAAACTGCATCCCTTAACTTGTTTTTCGTGTGCCTATTTTTTTGTGAATCGAATTCGA
GCTCGCCCCTCCTGACCACCTATNTGCATCAAGTGCCAAATGACCAGTCGAGTGTGCGGTTAGACAACTACTATACGGGC
AAGGAACTGGAGATTGAGTTGGATGTGGCTTTGACTCCTAGCCAAAATGCCCAGCGGTACTTCAAGAAGTACCAGAAACT
CAAGGAGGCGGTCAAGCACCTGA

Figure 6 E1

ivs 32, 35

ATATTTGCTCTCCTGCTCTTTAGGGGACAATGGAAAAAGTAGTCTGTATCCAACATTTTACAAAGTAGGATTTTTTCTAT
AAAATAGATTGTATATGACATTCAAATCCATTCTCAAACAACTCAAACTATTTGATTATATCTTAATCGGATTCACCCTA
GTTTTATCCTTTCTTCCAGCAATTTTTACCTACACACAACTGACAACAGATGCAAATGAGGCAAAAACAATTGCCTATGT
CCGCATCAATGGTGAGGTGGTCGACCAATTTGAATTATCAAAGGACACACCCCGTCAAGAAAAGACCTACTATCCCAATG
AAGGGCAATACAATATCATTGAAGTTGATGGCGAACGCATTCGTGTCAAGGAAGACAATAGCCCAGACCAAATCGCCGTT
ATG

Figure 6 F1

ivs 33

ACTCAGTTGAACGGAGTAGGATTTATAGGTAAATTGCCTCCAAATATCGTAAGACAATCCTCTATTGAAAAATAGGGGAT
TGTTTGTTTAGAAATAATGGTGGAGATTCTGTAAAAAGCGAAAGTGGTTGGAAAGTTAGGGTTTAGCCGAGAAAAAGAGA
CTTTTCTATCTATCTTTCACAATTTTCTGTCAATTTGTGGTAGAATAGAAAAAATAGATTTTTTATGAGGGATACCATGA
CATTAGTATATCAATCAACACGCGATGCTAAAAATACTGTATCGGCTAGTCAAGCGATTTTGCAGGGCTTGGCGACCGAC
GGTGGTTTGTTTACACCGCTTTCTATTCCAACAGTTGACTTGGATTTTTCTGTTTTGAAAGATGCTTCTTATCAAGACGT
T

Figure 6 G1

ivs 34

GTTTATCGTTCGCTGGAGGAAAAGGGCTATAATCCGATTAACCAAATCATTGGCTATGTATTAAGTGGGGACCCTGCTTA
TATTCCTCGCTATAATGATGCCCGCAATCAGATTCGTAAGCATGAACGAGATGAAATCATTGAAGAATTGGTGCGCTACT
ATTTGAAAGGGAATGGGATTGACCTCTAATGAGAATAATGGGATTAGACGTCGGTTCCAAGACAGTTGGTGTAGCCATTT
CAGATCCGTTAGGTTTCACGGCCCAAGGGTTGGAAATCATCCCAATCGATGAAGAAAAGGGCGAATTCGGTCTGGAGCGT
TTGACCGAACTTGTAGAACAGTACAAGGTTGATAAATTTGTTGTAGGCTTGCCGAAGAATATGAATAATACTAGTGGTCC
ACGT

Figure 6 H1

ivs 36

GGTATAATTATCTGATAAAAAACTTTGGAGACGACAGTGAGTTTAGAAAATTACATGCCGGATTTTGCCTTGGAAAAGGC
TTATGACGTGACCGTCGAAAGCTTGAAAAAACATGGCATAAAAGTAGTGTTTGTTGACTTGGATAATACCTTGATTGCTT
GGAATAATCCCGATGGTACGCCAGAGATGCGCCAGTGGTTACATGATTTGCAGGACGCAGGTATTCCTGTTGTGGTGGTG
TCTAACAATAAATACGAACGTGTCAAACGGGCGGTTGAAAAATTTGGGATTGAATTTGAAGCCTTCGCTCTCAAGCCTTT
CACCTTTGGGATTAACCGTGCTTTGAAACGCTTTGATGTCCAGCCGTATGAGGTAATTATGATT

Figure 6 I1

ivs 5, 10, 12, 22

ACGCACTTGCTCGCGTAGTCGATGAATTAGATGTACCCGTTATGGCTTTCGGTCTTAAAAATGATTTCCGAAATGAACTA
TTTGAAGGTTCCCAACATTTGCTCTTATTGGCTGATAAATTAGATGAAATCAAAACAATCTGCCAATATTGTTCTAAAAA
AGCGACAATGGTTTTGAGAACACAGGATGGAAAACCTACTTATGAAGGAGAACAAATCCAAATTGGTGGCAATGAAACCT
ACATTCCTGTCTGTCGCAAACATTATTTTTCACCAGAAATTAAAGATTTACCCTAATTTTTGAAAATGAAATGAGAAGCA
ACTGTAAACTGAGCAACTATATAGAACTGAATTTGCCTATGACTCTGTGCCAATTTTCATAACTTACATACTACGGCAAA
GGAATTGAACACG

Figure 6 J1

ivs 6, 7, 13, 14

GAAGGGATTAAACAATCCTATGCTATTCAGGCTGTTCGTGAAATTCGGATTATCGTTCATCCTAACAAGGTCACTGATGA
TCAGATTACCATCTTGGCCCATGATGTTCGTGAGAAAATTGAAAATAATCTGGATTATCCAGGAAATATCAAAATCACAG
TTATCCGTGAAACAAGAGCAACAGATGTTGCTAAGTAAATGTAGAAGAGTCCGAAGGGCTCTTTTTCTACTGGCTCAAAG
TTCGTTTTGGGTTGGGAATAGAAAATAGAAAATATTTTAATCGTATTTAAAAGCAGTTGAAATTCATGCTAAATTTTGTT
ACACTAGAATGAAAGATTTAAAAGGAGATATCATGAAAGAGCGAGGCTTACTCATTGTCTTTTCTGGTCCATCTGGTGCC
GGAAAAGGAACAGTTCGAAAGGAAATTT

Figure 6 K1

ivs 8

CTTCAAAGGACCCCAGGACCTTTGAATTCTCAAATACGCATCATGTTGACAGTTGCCACACCTACACCAAAATCAAATGC
CAACAAGCGTTGAGTCGGGTAATAGCGTAAGTAGCGCAAGGTCATGATAAGCTGCTCTTCCATACTTAGACGGCGTGGGC
GTCCTCCTTTTCGGTGTTGCTCTTGATAAGCGTCAGTGAGACAATCAAGCATCAGATGAAACGTCGCTTTTTTACACCTA
TCAACAATTTGAAATTCTCTGAGTTTAATTTTAAGACTTTTTCGTATGTTGTTTCCATACCTTTAGTATACCGCCTTTGA
GTTACCGAACAAGTCTATTGCTAAACTTGATGAAGGTTGTATTGTCTGTTATAATATTGGATA

Figure 6 L1

ivs 9, 17

GCCTATGAGACTCATTTTCCCTGTCTCAACTGCTCTAAGCAATTGTTACAGGTTGGTTGTAAGCGGGTTGTCTATATCAA
TGAATACCGCATGGATGACTATGCTCAGTACTTGTATAAAGAAAAGGGCTGTGAGTTGGTTCATTTGCCTCTAGAGGTGG
TTAAACAGGCATTTGCAGATGCCGAATTTATCTAATGATTTTGTAGAAGAGTGGTTGCATAGAACAACCCTCTTTATCTT
TAAGAAAATGCTAGGATAGTCGGTCAATCTATGCTATACTAGAAACGTTATTAAGTCCCGAAAAGGTAGTTTATAGACTA
GTTAATATTTGCAGAAACACTTGAAACACAATTAAAGAAACTGGTAATATTGAATAGTAAGCGTAAAAACTTTACTACAC
TTCAGTCACTATTTT